# MOLECULAR CHARACTERIZATION OF TOMATO MOTTLE VIRUS NONSTRUCTURAL PROTEIN GENES AND DEVELOPMENT OF TRANSGENIC PLANTS RESISTANT TO GEMINIVIRUSES

By

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KEY TO ABBREVIATION

| | |
|---|---|
| aa | amino acid |
| AbMV | abutilon mosaic virus |
| ACMV | African cassava mosaic virus |
| as | antisense |
| BCTV | beet curly top virus |
| BDMV | bean dwarf mosaic virus |
| BGMV | bean golden mosaic virus |
| bp | base pair |
| CabLCV | cabbage leaf curl virus |
| CaMV35S | cauliflower mosaic virus 35 S promoter |
| CMV | cucumber mosaic virus |
| CP | coat protein |
| CPMV | cowpea mosaic virus |
| C-terminus | carboxyl-terminus |
| DAI | days after inoculation |
| DSV | digitaria streak virus |
| ELISA | enzyme-linked immunosorbent assay |
| GUS | β-D-glucuronindase |
| IPTG | isopropyl-β-D-thiogalactopyranoside |
| IR | intergenic region |
| kb | kilobase |
| kDa | kilodalton |
| LB | Luria broth |
| MCS | multiple cloning site |
| β-ME | β-mercaptoethanol |
| MP | movement protein |
| dMP | defective movement protein |
| MPMR | movement protein-mediated resistance |
| MSV | maize streak virus |
| NOS | nopaline synthase |
| NPT II | neomycin phosphotransferase II |
| nt | nucleotide |
| N-terminus | amino-terminus |
| oligo dT | oligonucleotide deoxythymidine |
| PCR | polymerase chain reaction |
| PDR | pathogen-derived resistance |
| PYMV | potato yellow mosaic virus |
| PVY | potato virus Y |

| | |
|---|---|
| rbcS | rubisco small subunit |
| Rep protein | replication-associated protein |
| RT-PCR | reverse transcription- polymerase chain reaction |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SqLCV | squash leaf curl virus |
| T-DNA | transferred DNA |
| TEV | tobacco etch virus |
| TGMV | tomato golden mosaic virus |
| Ti-plasmid | tumor-inducing plasmid |
| TMV | tobacco mosaic virus |
| TMoV | tomato mottle virus |
| TYLCV | tomato yellow leaf curl virus |
| WDV | wheat dwarf virus |
| WT | wild type |
| X-Gal | 5-bromo-4-chloro-3-infolyl -β-D-galactopyranoside |

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# MOLECULAR CHARACTERIZATION OF THE NONSTRUCTURAL PROTEIN GENES OF TOMATO MOTTLE VIRUS AND DEVELOPMENT OF TRANSGENIC PLANTS RESISTANT TO THE VIRUS

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Tomato mottle virus (TMoV), a whitefly-transmitted geminivirus, is an important pathogen of tomato in Florida. The AC3, AC4, BC1 and BV1 open reading frames encoding the nonstructural proteins of TMoV were cloned and expressed in *Escherichia coli*. Polyclonal antiserum to each of these expressed proteins was prepared and used in Western blot analysis to index the expression of *AC3*, *AC4*, *BC1* and *BV1* genes in TMoV-infected and transgenic plants. The BC1 movement protein (MP) was detected in the extracts from both infected plants and transgenic plants. The BV1 MP was detected in the extracts from infected plants, and appeared to accumulate as leaves aged in contrast to the BC1 MP. The AC3 and AC4 proteins were also detected in the extracts from infected plants.

Transgenic tobacco plants were generated by *Agrobacterium*-mediated transformation with the *AC4*, *BC1* and *BV1* gene constructs. Transgenic plants expressing the BC1 protein had a range of phenotypes from severe stunting and leaf mottling to no visible symptoms. Three different mutated forms of the *BC1* gene were identified from these transgenic plants. Two mutated forms (*BC1A* and *BC1At/r*) were associated with asymptomatic phenotypes, whereas the third one (*BC1S*) was associated with the phenotypes that were more severe than those of TMoV infection. The *BC1At/r* mutant had an open reading frame with a deletion of 119 amino acid residues at the C-terminus of the BC1 and an addition of 26 amino acid residues from an unknown source. This asymptomatic *BC1At/r* suppressed the symptom-inducing effects of the symptomatic *BC1S* in tobacco plants containing both of these genes.

Transgenic plants were evaluated for resistance to geminiviruses and to several RNA viruses. Plants expressing the mutated *BC1* with asymptomatic phenotypes were found to be resistant to TMoV and cabbage leaf curl geminiviruses, whereas plants with symptomatic phenotypes accentuate systemic infection of tobacco mosaic tobamovirus or cucumber mosaic cucumovirus. The symptom suppression and broad spectrum resistance mediated by the defective BC1 movement protein may involve trans-dominant, negative-interference.

The spontaneously mutated *BC1* genes (*BC1A and BC1At/r*) identified in this study may be used as novel resistance genes for the control of geminivirus diseases.

# CHAPTER 1
# INTRODUCTION

Tomato mottle virus (TMoV), a typical bipartite geminivirus (Abouzid et al., 1992b), infects tomato and other dicotyledonous plants. This virus is transmitted by the whitefly, *Bemisia tabaci* (Gennadius) and causes significant loss of yield in infected tomato plants by inducing vein chlorosis, leaf mottling and plant stunting (Polston et al., 1993).

## General Characteristics of Geminiviruses

Geminiviruses are of agronomic importance throughout the tropical and subtropical regions of the world. Since their discovery as DNA plant viruses in the 1970s (Goodman, 1977; Harrison et al., 1977), geminiviruses have been recognized not only as the cause of serious and increasingly frequent diseases of crops around the world , but also as useful models for studies of DNA replication and cell processes, and as potential vectors for the expression of foreign genes in plants (Lazarowitz, 1992; Hiebert et al., 1996). As a result, geminiviruses have received increasing research attention. So far, more than 60 different geminiviruses have been identified in the world with about a dozen infecting tomato (Briddon & Markham, 1995). The most economically important geminiviruses are the whitefly-transmitted African cassava mosaic virus (ACMV), tomato

yellow leaf curl viruses (TYLCV), and the leafhopper-transmitted maize streak virus (MSV) (Fauquet & Fargette, 1990; Brown & Bird, 1992; Thottappilly, 1992).

The detailed genetic and genomic information for a number of distinct geminiviruses led the International Committee for the Taxonomy of Viruses to recognize the family, Geminiviridae (Briddon & Markham, 1995). Based on their hosts, vector specificity and genome organization, three genera are currently recognized. Genus "Subgroup I Geminivirus"(Genus I) consists of monocot-infecting (with the exception of tobacco yellow dwarf virus), leafhopper-transmitted viruses with monopartite genomes and narrow host ranges. There are 12 species and 2 tentative species in this genus. Genus "Subgroup II Geminivirus"(Genus II) consists of dicot-infecting, leafhopper-transmitted (with the exception of tomato pseudo-curly top virus) viruses with monopartite genomes. The genus contains type species beet curly top virus (BCTV) and two other tentative species. The BCTV has a very wide host range, infecting over 300 species in 44 plant families. Genus "Subgroup III Geminivirus" (Genus III) is the largest group, containing 43 species and 9 tentative species. All viruses in Genus III are dicot-infecting and whitefly-transmitted with relatively narrow host ranges, and most of them contain a bipartite genome (except for TYLCV and TYCV) (Briddon & Markham, 1995).

Genome Organization

The twinned particle morphology of geminiviruses is a unique feature, making them distinct from all other viruses. The incomplete icosahedral virions (about 18 x 30 nm) contain either a monopartite or bipartite genome with single-stranded (ss) circular

DNA (Briddon & Markham, 1995). The genes in geminiviruses are arranged in two divergent overlapping clusters separated by an intergenic region of about 200 nucleotides (nt). The intergenic region of all geminiviruses contains a GC-rich inverted repeat sequence that has the potential to form a stem-loop structure (Lazarowitz, 1992). The inverted repeats flank an 11 to 16 base AT-rich sequence containing the nonamer motif TAATATTAC that is present in all genimiviruses (Laufs et al., 1995a).

Monopartite genome. Although monopartite geminiviruses fall into three different genera, they have a similar genomic organization in a 2.5-3.0 kb circular ssDNA. Genus I geminiviruses contain four open reading frames (ORFs), C1, C2 , V1 and V2. Viruses from Genus III such as tomato yellow leaf curl virus-Israel, have similar genome organizations (containing C1, C2, C3, C4, V1, and V2 ORFs) to that of the Genus II geminivirus, BCTV, except the latter contains another overlapping ORF V3.

Bipartite genome. Bipartite geminiviruses contain two genomic components, designated as DNAs A and B, each 2.5-2.8 kb ssDNA. Four proteins encoded by the A component are required for DNA replication and encapsidation, while the B component codes for two proteins required for systemic movement in infected plants (Brough et al., 1988; Etessami et al., 1988; Stanley, 1991; Noueiry et al., 1994; Pascal et al., 1994; Smith and Maxwell, 1994). The ~200 nt intergenic region (IR; also known as common region) is identical in sequence between the A and B components of a bipartite geminivirus but is distinct in sequence among different geminiviruses (Davis et al., 1987; Lazarowitz, 1992).

Viral Replication

Geminiivirus genomes replicate to high copy number in the nuclei of infected cells via a double-strand (ds) intermediate by a rolling circle mechanism analogous to that used by ssDNA phage and plasmid (Saunders et al., 1991; Stenger et al., 1991; Lazarowitz, 1992). Upon entry into the host cell nucleus, the viral ssDNA is believed to be converted to a ds form. In monocot-infecting geminiviruses, a ~80 nt DNA fragment binds tightly to the small intergenic region (IRs) and putatively functions as a primer for viral DNA synthesis *in vitro* (Donson et al., 1984; Hayes et al., 1988). In bipartite geminiviruses, a *cis* sequence required for complementary-strand synthesis is located within the IR, but no putative primer DNA is found in the virion (Mullineaux et al., 1992; Timmermans et al., 1994).

After conversion into the ds molecule, the geminivirus genome is transcribed, and viral proteins are synthesized. The replication-associated protein (Rep protein) (C1/AC1 or C1:C2) encoded by geminiviruses and the hairpin structure in the IR are essential for geminivirus replication (Laufs et al., 1995b; Orozco & Hanley-Bowdoin, 1995). The replication is initiated between position +7 and +8 of the conserved sequence 5'-TAATATTAC, which is cleaved by Rep protein C1/AC1 (Heyraud et al., 1993; Stanley, 1995). Rep protein preferentially binds to ssDNA at IR and functions *in vitro* as a site-specific endonuclease that nicks the ssDNA at the loop site of the hairpin structure for the initiation of replication (Heyraud-Nistchke et al., 1995; Laufs et al., 1995b; Orozco & Hanley-Bowdoin, 1995).

The initiation and termination sites for positive-strand DNA synthesis have been mapped to the hairpin region for members of all three geminivirus groups (Heyraud et al., 1993; Stanley, 1995). During the replication of tomato golden mosaic virus (TGMV), the hairpin structure appears to be required for both initiation and termination of positive-strand DNA synthesis, whereas during the replication of wheat dwarf virus (WDV), the structure may only be required for termination of positive-strand synthesis (Orozco & Hanley-Bowdoin, 1995). The C3/AC3 protein is required for efficient replication of Genus III geminiviruses (Sunter & Bisaro, 1992). In contrast, Genus I geminiviruses do not encode C3 homologues, and lack a directly repeated motif for C1 binding. Release by the AC1 protein of the ssDNA segment from an integrated DNA flanked by two geminivirus hairpin-loops results in formation of a long ss gap that is believed to be filled by host repair enzymes.

Transcription

Geminivirus genomes are transcribed bidirectionally, and transcription initiates within the IR and either side of the IR. The genes of these viruses are divergently transcribed to terminate at polyadenylation sites in the small IR (Townsend et al., 1985; Sunter & Bisaro, 1989; Hanley-Bowdoin et al., 1988; Frischmuth et al., 1991). The transcripts corresponding to most of the genes of the bipartite geminiviruses, TGMV, African cassava mosaic virus (ACMV) and abutilon mosaic virus (AbMV), have been detected in infected plants and identified *in vitro* by the analyses of Northern blot hybridization, S1 nuclease protection assay, primer extension and sequencing (Sunter et

al., 1989; Hanley-Bowdoin et al., 1989; Frischmuth et al., 1991). The transcripts for virion sense genes appear to be a single-transcript for each gene, whereas more than one transcripts are identified for gene(s) whose ORFs are in the complementary sense of bipartite geminiviruses.

Sunter and Bisaro (1989) demonstrated that both the AC1 and BC1 complementary sense transcripts were a family of distinct, 3' coterminal RNA species with different 5' ends. The AC1 RNAs of TGMV comprise a family of transcripts with 5' ends that map to nts 62, 2540, 2548 and 2515 (Hanley-bowdoin et al., 1988; Sunter and Bisaro, 1989). Three transcripts of the TGMV BC1 gene have 5' ends that map to nts 62, 2499 and 2440 near the IR of DNA B. However, only two transcripts of the BC1 gene are identified in AbMV, with 5' ends at nts 72 and 2353 (Frischmuth et al., 1991). Although different start sites are identified in the BC1 transcripts, all of the transcripts of both viruses are coterminal at their 3' ends and appear to share a common polyadenylation signal. The consensus sequence AATAAA has been shown to be a termination signal for plant mRNAs (Joshi, 1987). A single base substitution in the hexanucleotide consensus sequence may result in aberrant or inefficient processing of the 3' terminus in animal cells (Proudfoot & Brownlee, 1976). Surprisingly, more polyadenylation signal motifs are present in the complementary than the viral sense of DNA A and DNA B (Frischmuth et al., 1991). The overlap of 50-75 nt at 3' ends of the viral and complementary sense transcription units of TGMV DNA A and DNA B may play a role in the regulation of viral gene expression (Sunter et al., 1989).

In contrast to the lack of evidence for splicing of transcripts in the bipartite viruses, transcript splicing has been found in the complementary sense of monopartite viruses, maize streak virus (MSV), Digitaria steak virus (DSV) and WDV, and a functional intron has been located at the junction between C1 and C2 (Accotto et al., 1989; Schalk et al., 1989; Mullineaux et al., 1990; Dekker et al., 1991). In case of virion sense genes, two overlapping transcripts coterminal at 3' ends have been identified in MSV, WDV and DSV (Morris-Krsinich et al., 1985; Accotto et al., 1989; Schalk et al., 1989). However, working with a Czech isolate of WDV, Dekker et al. (1991) detected only one transcript that spans both V1 and V2 ORFs.

Gene Function(s)

Most gene functions of geminiviruses either have been characterized or proposed. The Rep protein, essential for viral replication, has multiple functions such as ATPase and site-specific endonuclease, helicase, ssDNA joining, and specific binding to dsDNA activities (Fontes et al., 1992, 1994; Thommes et al., 1993; Desbiez et al., 1995; Laufs et. al., 1995a, 1995b; Orozco & Hanley-Bowdoin, 1995). The Rep protein is also involved in gene regulation such as repressing its own synthesis at the level of transcription (Haley et al., 1992; Sunter et al., 1993). The AC2 protein facilitates the expression of both the AV1 and BV1 genes at the transcriptional level in TGMV, serving as a transactivator to activate the promoters of both genes (Sunter & Bisaro, 1992). The AC3 protein is required for efficient DNA accumulation and infection by enhancing viral replication (Elmer et al., 1988; Sunter et al., 1990). The AC4 protein has only been detected in *in*

*vitro* translation products (Thommes & Buck, 1994). Using GUS-NEO expression system, Groning et al. (1994) reported that ORF AC4 has a suppressive effect on expression of the *AC1* gene of TGMV. However, mutational analyses indicate that the ORF AC4 of TGMV or of potato yellow mosaic virus (PYMV) does not affect virus replication or symptom development (Elmer et al., 1988; Sung and Coutts, 1995). Furthermore, transient expression of the *AC4* does not affect the *AC1* expression of ACMV in *N. tabacum* protoplasts (Hong & Stanley, 1995). In contrast, C4 protein encoded by the monopartite geminiviruses, BCTV and TYLCV has been demonstrated to be involved in virus movement and a determinant of symptom severity (Stanley & Latham, 1992; Jupin et al., 1994; Rigden et al., 1994). The CPs are essential for systemic infection of monopartite geminiviruses (Lazarowitz et al., 1989; Briddon et al., 1989; Woolston et al., 1989; Rigden et al., 1993), but the CPs may only be required for the systemic infection of bipartite geminiviruses when these viruses infect poorly adapted hosts (Pooma et al., 1996).

Two proteins, BC1 and BV1 (also known as BL1 and BR1 in the literature), encoded by the DNA B of bipartite geminiviruses are required for viral infectivity and systemic infection (Brough et al., 1988; Etessami et al., 1988; Von Arnim & Stanley, 1992b; Smith & Maxwell, 1994; Sung & Coutts; 1995; Haley et al., 1995; Ingham et al., 1995). Recent mutagenesis studies on these movement proteins (MPs) of the bipartite geminiviruses, bean golden mosaic virus (BGMV), squash leaf curl virus (SqLCV), ACMV, and PYMV not only confirmed that the MPs are essential for viral infectivity and systemic infection, but also revealed the potential functional domains and the tolerance of

these viruses to changes in these MPs. Most mutations at conserved amino acid (aa) residues or regions impair the function of the MPs but the mutations at the C-terminus are less sensitive than those at the N-terminus of BC1. The virus may tolerate the alteration of a conservative aa (functional analog) residue better than that of a non-conservative one (Smith and Maxwell, 1994; Haley et al., 1995; Ingham et al., 1995; Sung & Coutts, 1995).

Smith and Maxwell (1994) demonstrated that mutant pGABL1-1 of BGMV failed to cause systemic infection because the mutation resulted in a frameshift and a putatively truncated BC1 protein consisting of the 123 aa N-terminus and a 22 aa fusion. The mutant pGABL1-4 ($H^{102}$ to $E^{102}$) caused very attenuated symptoms, and one plant had a symptomless infection. It was postulated that this mutation (substitution of Glu for His in a hydrophobic region of the protein) may cause a change from a $\beta$ sheet to an $\alpha$ helix in the area of the predicted protein secondary structure. The pGABL1-3 ($D^{248}$ to $H^{248}$) and pGABL1-5 ($M^{217}$ to $S^{217}$) mutants caused either a slight attenuation of symptoms or wild-type (WT) symptoms although the nucleotide substitution in pGABL1-3 was in a region of highly conserved aa sequence near the C-terminus of the protein. Sung and Coutts (1995) found that a one aa (serine) insertion in near the N-terminus of the PYMV BL1 caused the virus to lose its infectivity to *Nicotiana benthamiana.*

Haley et al. (1995) reported that seven out of eight point mutations (substitutions) introduced into the BC1 ORF of ACMV prevented the virus constructs from being infectious. These point mutations were among the conserved amino acids, which were pBC52V ( Asp to Val), pBC52G (Asp to Gly), pBC100Y (His to Tyr), pBC100D (His to Asp), pBC145I (Lys to Ile), pBC206V (Gly to Val), and pBC206E (Gly to Glu). In

contrast to pBC145I (Lys to Ile), the mutant pBC145R (Lys to Arg) retained its infectivity in *N. benthamiana* and *N. clevelandii,* but lost infectivity to *N. tabacum* cv. Samsun, indicating that the virus was able to tolerate a conservative change.

Based on the infectivity of SqLCV clones mutated in the BC1 ORF, Ingham et al. (1995) devided the mutants into three classes. Class I mutants caused WT SqLCV symptoms and retained highly infectious in both cucurbit hosts. Class II mutants were 40-75% as infectious as WT SqLCV in pumpkin and 11-50% as infectious in squash, and all Class II mutants were characterized by a delay of symptom development and attenuated systemic disease symptoms in both cucurbit hosts. These class II mutants included the alanine substitution mutations $F^{35}$, $K^{140}/K^{142}$, and $N^{67}$ and $N^{260}$ (two potential glycosylation site), and the 100 aa C-terminal truncation mutant Δ 194-293. Class III mutants had little to no infectivity in either cucurbit host, indicating that a severe impairment occurred in these mutants. The mutations were the two internal deletions Δ 11-23 and Δ 160-169, and alanine substitution mutations $D^{78}/R^{80}$ and $W^{208}/K^{211}$, all of which are in the well-conserved regions of the BC1 among bipartite geminiviruses.

By sequence comparisons of SqLCV-E and SqLCV-R strains, Ingham and Lazarowitz (1993) identified three missense mutations ($Pro^{9}$ to His, $Asn^{10}$ to Tyr , and $Arg^{98}$ to Cys) in the BV1 protein of the SqLCV-R mutant that has lost the ability to infect *N. benthamiana*. Using site-directed mutagenesis, they confirmed that only one mutation ($Arg^{98}$ to Cys) was responsible for the alteration of host range. Futhermore, mutational analysis revealed that both BV1 and BC1 proteins are involved in the determination of

host range of SqLCV, but only the BC1 protein was related to viral pathogenicity (Ingham et al., 1995).

The movement proteins of plant viruses are essential for cell to cell spread of infection, and in part, determine the host range and pathogenesis (Heinlein et al., 1995). The pathogenic properties of bipartite geminivirus BC1 proteins have been demonstrated not only by mutational analysis but also by transgenic plant studies. Transgenic plants expressing the SqLCV BC1 protein display typical SqLCV disease symptoms, suggesting that the growth and development defects may result from misregulated phloem transport, which may be caused by the interaction between BC1 protein and host proteins (Pascal et al., 1994). However, the molecular and physiological mechanisms of the effects of the BC1 protein on the alteration of host metabolism remain to be investigated.

Virus Movement

The distribution of geminiviruses under natural field conditions is primarily determined by epidemiological constraints imposed on the whitefly and leafhopper vectors (Frischmuth & Stanley, 1993). Geminivirus movement within a plant and spread from plant to plant require the participation of the movement proteins (MPs) and/or coat proteins encoded by these viruses. The development of systemic infection of plant viruses involves two distinct types of movement, cell-to-cell and long distance transport via the vascular system. The intercellular movement of most, if not all, plant viruses requires virus encoded protein(s). The movement proteins encoded by plant viruses are essential for infection of the host plant but are not required for viral replication or encapsidation

(Hull, 1989; Maule, 1991). For cell-to-cell movement, there are two different mechanisms that illustrate how plant viruses conduct an active process for intercellular movement facilitated by their movement proteins. One mechanism, exemplified by tobacco mosaic virus (TMV) (Wolf et al., 1989; Citovsky et al., 1990), indicates the movement of viruses as a nucleoprotein complex through plasmodesmata. The other mechanism, exemplified by cowpea mosaic virus (CPMV) (Van Lent, et al., 1990; 1991), involves a tubular structure formed by a movement protein, through which virions are moved from cell to cell.

Geminiviruses, unlike RNA plant viruses, replicate and accumulate in the cell nucleus rather than in the cytoplasm. Prior to moving to adjacent cells, the virus requires a DNA binding protein and a means to transport of the viral genome in and out of the nuclei (Lazarowitz, 1992). The BC1 (BL1) and BV1(BR1) proteins encoded by DNA-B of the bipartite geminiviruses, bean dwarf mosaic virus (BDMV) and SqLCV coordinate the movement of geminivirus DNA in and out of nuclei and from cell to cell. Using microinjection of the *in vitro* expressed BDMV MPs labeled with fluroescein isothiocyanate, Noueiry et al. (1994) observed that the BC1 MP increased mesophyll plasmodesmal size and potentated the movement of dsDNA from cell to cell, while the BV1 MP mediates the movement of dsDNA out of the nucleus, and suggested that two MPs may coordinate the movement of viral DNA across both nuclear and plasmodesmal boundaries. The BC1 and BV1 MPs of SqLCV have also been demonstrated to coordinate the movement of viral DNA across both nuclear and cell wall boundaries. However, the SqLCV BV1 MP binds ssDNA, functions as a nuclear shuttling protein, and

contains domains essential for interaction with BC1 and nuclear localization, whereas the SqLCV BC1 MP has very weak affinity for ssDNA and no apparent ability to bind dsDNA. In addition, the SqLCV BC1 MP not only localizes itself to the cell-periphery, but also brings SqLCV BV1 MP to the cell periphery through protein-protein interaction, indicating that the BC1 may provide directionality to the movement of viral genome (Pascal et al., 1994, Sanderfoot & Lazarowitz, 1995; Sanderfoot et al., 1996).

A model for the movement of SqLCV proposed by Sanderfoot & Lazarowitz (1995) predicts that BV1 is a nuclear shuttle protein that binds ssDNA and moves it to the cell periphery where, as the result of BC1 action, the BV1-ssDNA complex moves locally to adjacent uninfected cells and also enters the sieve elements, from which the BV1-ssDNA complex may initiate infection at distal sites along the phloem, and this movement is facilitated by the direct interaction of both BC1 and BV1 MPs.

## Theory and Application of Transgenic Plants with Viral Resistance

Advances in molecular biology and plant transformation and regeneration technologies have made it possible to genetically engineer plants with foreign gene(s). One of the major objectives for transgenic plant development is crop resistance to pests such as viruses, insects, bacteria, fungi and nematodes.

### Development of Transgenic Plants

The development of transgenic plants has exploited a variety of techniques including *Agrobacterium*-mediated transformation (Horsch et al., 1985; An et al., 1985)

and direct gene transfer, such as particle bombardment (Klein et al., 1987; Sanford, 1988), electroporation (Fromm et al., 1986), laser microbeam injection (Weber et al., 1990), pollen and embryo DNA imbibition (Hess, 1987; Topfer et al., 1990).

*Agrobacterium*-mediated transformation is a widely used method for inserting genes into plant genomes for both basic and applied studies (Martineau et al., 1994). An increasing number of transformation vectors and strains of *Agrobacterium tumefaciens* have been developed and used in recent years. Because of their small size, easy manipulation and maintenance, the transformation vectors often used are binary in nature, containing origins for replication in *Agrobacterium* and *Escherichia coli*, and an antibiotic resistance gene for the maintenance of the plasmid in bacterium. The key component of the expression vector is an expression cassette flanked by the left and right borders (two imperfect 25 bp repeats, derived from the Ti plasmid of *Agrobacterium*). The expression cassette includes cloning site(s) for foreign gene(s) and a selective marker gene, which only expresses in plant cells. After cloning a foreign gene into the cloning site under the control of a certain promoter such as CaMV 35S, the expression vector can be multiplied in *E. coli* and then mobilized into an *Agrobacterium* strain harboring a disarmed Ti plasmid (D-Ti) with a deletion of T-DNA, and thereafter used for transformation of plant tissue or explants. The products of *vir* genes located in the D-Ti recognize the border sequences, and coordinately transfer the expression cassette into the plant chromosome(s). The expression of the selective marker gene (encoding for antibiotic resistance) allows selection of transformed plant cells on antibiotics-amended medium during plant regeneration (Joshi & Joshi, 1991).

Transgenic Resistance to Plant Viruses

Viruses cause serious losses to crop production around the world. The losses result not only from virus diseases themselves but also from the costs of insecticides and chemicals used for vector control (Hull & Davies, 1992). The development of virus-resistant cultivars through conventional breeding programs, although effective, often proves to be difficult, time consuming, and the resistance can be overcome by rapid change of the adaptation to host in the virus population (Frischmuth & Stanley, 1993; Lomonossoff, 1995). An alternative way to produce virus resistance is so-called nonconventional breeding by which virus resistance may be developed in transgenic plants transformed with viral sequence(s). This phenomenon is known as "pathogen-derived resistance" (PDR). The theory of PDR implies that the transformation of a susceptible plant with genes derived from a pathogen's own genetic material may result in the development of heritable resistance in the plants (Sanford and Johnson, 1985).

The first achievement of PDR was demonstrated by Powell-Abel et al. (1986), who reported that transgenic tobacco plants expressing TMV-CP were resistant to the virus. This discovery opened a new field of plant science research, and activity in this aspect of research has expanded rapidly in the past decade. Many studies have been conducted to determine what viral sequences could confer resistance and to investigate the potential mechanisms for these PDRs. It appears that any part of a plant viral genome can potentially give rise to PDR (Lomonossoff, 1995). A number of hypotheses have been proposed for the mechanisms of PDR from CP, replicase, RNA, defective MP, antisense sequences, satellites, defective interfering molecules and other nonstructural genes or

intergenic region sequences (reviewed by Hull & Davies, 1992; Frischmuth & Stanley, 1993; Lindbo et al., 1993a; Kavanagh & Spillane, 1995; Lomonossoff, 1995). It is conceivable that multiple mechanisms may be involved, and that they operate with different efficiencies in different transgenic plants and host-pathogen systems. (Lindbo et al., 1993a). The resistance derived from a single viral gene in a single plant species may also involve more than one mechanism and may inhibit several different stages in the process of virus infection, replication or movement (Fitchen & Beachy, 1993). Although movement protein-mediated resistance (MPMR) has received much less attention than other PDR, such as coat protein-mediated resistance, recent studies indicate promising prospects for this approach (Lapidot et al., 1993; Beck et al., 1994; Cooper et al., 1995).

Movement Protein-Mediated Resistance (MPMR)

Due to their special functions, the viral MPs may be novel gene sources for PDR (Deom et al., 1992; von Arnim & Stanley, 1992b; Frischmuth & Stanley, 1993). The first example of MPMR was demonstrated by Malyshenko et al. (1993), who described that transgenic plants expressing TMV tsMP (a temperature sensitive mutation in the MP) had reduced rates of TMV infection when these plants were held at the nonpermissive temperature. In addition, tobacco plants (nonpermissive host) expressing the 32 kDa MP of brome mosaic virus were resistant to TMV. Lapidot et al. (1993) reported that transgenic tobacco plants expressing a defective movement protein (dMP) of TMV with a deletion of N-proximal aas 3, 4, and 5 were resistant to TMV and two other tobamoviruses, tobacco mild green mosaic virus and sunnhemp mosaic virus.

Subsequently, Cooper et al. (1995) demonstrated that the same dMP transgenic plants were resistant to tobacco rattle tobravirus, tobacco ringspot nepovirus, alfalfa mosaic alfamovirus, peanut chlorotic streak caulimovirus, and cucumber mosaic cucumovirus (CMV), whereas the functional analog increased host susceptibility. The most promising example was demonstrated by Beck et al. (1994), who reported that transgenic plants expressing a 13 kDa dMP mutated in a conserved region of all the viruses possessing triple-gene block were highly resistant to O, M, and J strains of white clover mosaic virus, to the other two potexviruses, potato virus X and narcissus mosaic virus, and to the carlarvirus potato virus S. Transgenic tobacco plants carrying the 48 kDa MP gene of CPMV were highly resistant to Sb, S1, and S8 strains of CPMV, but not to other comovirus species such as cowpea severe mosaic virus (Sijen et al., 1995).

These examples indicate that plants expressing MP or dMP may result in a broad-spectrum resistance, and that dMPMR interferes with the spread of infecting virus but not with virus infection. The TMV dMPMR, although not complete, restricts the local spread of tobamoviruses, and retards the systemic infection of heterologous viruses by delaying symptom expression and/ or reducing accumulation of virus in upper leaves in transgenic plants (Cooper et al., 1995). Based on the fact that transgenic plants expressing wild type TMV MP have increased their susceptibility, it is suggested that it is better not to use a wild type MP gene for PDR since plants expressing such a gene are likely to have increased susceptibility to certain other viruses (Cooper et al., 1995).

The cellular and molecular mechanisms of dMPMR are not clear, but it was proposed that dMPMR prevents the accumulation of the MP of the challenging virus in

such a way as trans-dominant negative interference (Lapidot et al., 1993; Beck et al., 1994). This is an application of a concept called "dominant negative mutation", which was described by Herskowitz (1987) as the inhibition of the function of a wild-type gene product by an overproduction of the inhibitory variant of the same gene product. Because of the multifunctional properties of a MP, the interference may involve any aspect of the MP-associated functions such as multiprotein interaction, subcellular targeting to cell wall or to plasmodesmata, nucleic acid binding, or modification of plasmodesmatal structure and/or function (Lapidot et al., 1993; Cooper et al., 1995). In the scenario of TMV dMPMR, the dMP in transgenic plants reduces, but does not eliminate virus spread, suggesting that this may be the result of incomplete interference or incomplete competition (Lapidot et al., 1993). In contrast to TMV dMPMR, the resistance derived from the CPMV 48 kDa MP gene specifically inhibits the replication of RNA2 at the cellular level, suggesting such resistance is RNA-mediated (Sijen et al., 1995).

RNA-Mediated Resistance

In general, RNA-based PDR includes sense RNA-, antisense RNA- and satellite RNA-mediated resistance. The potential mechanism of RNA-mediated resistance is well illustrated on the basis of sense RNA-mediated virus resistance. Plants expressing a transgene derived from an RNA virus can be completely resistant to the virus. This resistance is present in both translatable and untranslatable constructs, indicating that the translation product of the transgene transcript is not necessarily involved in eliciting resistance (De Haan et al., 1992; Lindbo and Dougherty, 1992a, 1992b; van der Vlugt et

al., 1992; Pang et al., 1993). Lindbo et al (1993b) reported that transgenic tobacco plants expressing a full-length or truncated form of the tobacco etch virus (TEV) CP gene can recover from TEV infection. The molecular study of this phenomenon revealed that the steady state transcripts of the TEV-CP transgene in the "recovered tissue" is reduced by 12-22 fold as compared with that in uninoculated transgenic tissue, whereas the rate of transgene transcription is not different between the recovered tissue and uninoculated tissue in nuclear runoff assays. These observations led to the conclusion that the antiviral state found in recovered tissue is due to the induction of a specific cellular RNA-degradation system. Subsequent studies provided more evidence that transgenic plants displaying RNA-mediated resistance phenotypes transcribe the transgene(s) at a high rate but accumulate the transgene transcript at low levels. Furthermore, transcript level is inversely correlated with resistance, and untranslatable constructs give rise more frequently to high level resistance (Dougherty et al., 1994; Smith et al., 1994; Muller et al., 1995; Swaney et al., 1995; Goodwin et al., 1996).

Although the nature of the RNA-degrading mechanism mentioned above is unclear, the RNA degradation occurs in cytoplasm, and the induction of this degradation system is controlled by transgene copy number in a dosage-dependent fashion (Goodwin et al., 1996). For the highly resistant state, it is necessary to have three or more copies of a transgene, whereas only one or two copies of the transgene may elicit resistance, such as recovery. The threshold model proposed by Lindbo et al. (1993b) and Smith et al. (1994) suggests that the excess levels of transgene transcripts activate a post-transcriptional surveillance system that specifically targets an RNA sequence via cleavage of specific sites

within the targeted sequence, resulting in the elimination of both viral RNA and transgene transcript in the cytoplasm (Lindbo et al., 1993b; Goodwin et al., 1996).

In contrast to protein-mediated resistance, RNAMR generally provides high-level but highly specific resistance. The higher levels of resistance obtained with RNAMR seem to be of more practical use. However, this type of resistance may be overcome more easily in a field owing to variation in the virus population (Lomonossoff, 1995).

Transgene Expression and Regulation

The expression of foreign genes in transgenic plants shows varying levels of expressivity in different lines or in siblings with a given transgenic line (Meyer, 1995). Gene "silencing' is a general phenomenon observed in transgenic plant studies. This phenomenon occurs frequently when there are multiple copies of a particular sequence present in the genome. The presence of homologous sequence not only affects the stability of transgene expression, but also may alter the activity of the endogenous gene after insertion of a homologous sequence into the genome (Jorgensen, 1990; Napoli et al., 1990; Smith et al., 1990; van der Krol et al., 1990; Hobbs et al., 1993; Dehio & Schell, 1994; Matzke et al., 1994). This homology-dependent gene "silencing" phenomenon is known as cosuppression (trans-interference between endogenous genes and/or introduced transgenes) (Jorgensen, 1990), and is often related to gene inactivation at transcriptional or post-transcriptional levels (Brusslan et al., 1993; Meyer et al., 1993; Dehio & Schell, 1994;. Smith et al., 1994; Muller et al., 1995). Various suggestions or models to explain the phenomena of gene "silencing" include methylation, cosuppression, paramutation,

alteration of chromatin structure, and positional effect (reviewed by Jorgensen, 1992; Finnegan & McElroy, 1994; Flavell, 1994; Matzke &Matzke, 1993; Meyer & Saedler, 1996).

Varying levels of resistance in different transgenic plant lines transformed with the same gene appear to be the norm in pathogen-derived resistance studies (Longstaff et al., 1993; Hull, 1994; de Feyter et al., 1996). Due to the random nature of transgene insertion into the host chromosome, transgenic lines may have the transgene incorporated into different locations with a different copy number. There is no general correlation between levels of resistance and levels of gene expression. In some cases there is positive correlation, and in others there is a lack of positive correlation, or even a negative correlation (reviewed by Hull & Davies, 1992; Fitchen & Beachy, 1993; Lindbo et al, 1993a; Kavanagh & Spillane, 1995; Lomonossoff, 1995). Even in the double-haploid isogenic transgenic tobacco lines, which theoretically should be identical in terms of copy number and organization of the transgene, the lines displaying high levels of resistance always give rise to a proportion of susceptible progeny (Smith et al., 1994). Therefore, one should not necessarily expect a consistent resistance phenotype even in homozygous transgenic plants (Lomonossoff, 1995).

Engineering Geminivirus Resistance in Transgenic Plants

In light of successful PDR for plant RNA viruses, a number of strategies involving defective DNA, antisense sequence, coat protein and replication-associated protein AC1 have been investigated for their potential in genetic engineering geminivirus resistance (Stanley et al., 1990; Day et al., 1991; Stenger, 1994; Kunik et al., 1994; Bejarano &

Lichtenstein, 1994; Hong & Stanley, 1996). Stanley et al. (1990) first reported that transgenic plants carrying defective viral DNA attenuated disease symptoms of its cognate geminivirus ACMV infection. However, since then, only a few examples of geminivirus resistance have been reported in transgenic plant studies, and most of these examples illustrate incomplete resistance, involving either a delay in the development of disease or attenuation of the disease symptoms.

CP-mediated resistance or interference with vector transmission. Kunik et al. (1994) reported that transgenic tomato plants expressing viral sense sequence (V1 and V2 ORFs) of TYLCV were resistant to the virus. Although the V1 RNA could be detected in all transformed lines, only six lines express detectable CP (V1) protein and conferred resistance to the virus. The levels of resistance were positively correlated with levels of the V1 mRNA and protein. The resistance was manifested by a delay of symptom development and by remission from virus infection, and the remission was increased with repeated inoculation. Due to the constructs containing both V1 and V2 ORFs and lack of analysis of the expression of V2 ORF, whether the resistance is mediated by V1 or V2 or both together remains to be determined.

The coat protein of geminiviruses has been demonstrated to be associated with insect vector-mediated transmission (Briddon et al., 1990; Azzam et al., 1994; Brown et al., 1996). The strategy of transforming plants with dysfunctional CP was proposed for interference with vector-mediated geminivirus transmission (Brown et al., 1996).

Defective DNA interference. The defective genome components are related to, and depend on the parent virus for their replication, and they are referred to as defective

interfering (DI) molecules if they disrupt or enhance virus replication (Holland, 1990). Stanley and Townsend (1985) identified a natural defective mutant of ACMV DNA B with deletion of the entire BV1 gene and disruption of the carboxy-terminus of the BC1 gene. This defective mutant interferes with virus proliferation when coinoculated to plants with viral DNA. Furthermore, transgenic tobacco plants carrying a tandem repeat of this DI DNA ameliorate symptoms of ACMV infection, and show protection only to isolates of ACMV but not to the related viruses, TGMV and BCTV (Stanley et al., 1990). The symptom amelioration is associated with disproportionate reductions in the rates of 20% for DNA A and 70% for DNA B compared with those in controls. Since the symptom-inducing element(s) is mapped in DNA B, the drastic reduction of DNA B rather than the slight decrease of DNA A is believed to be the primary cause of symptom amelioration. The DI DNA interference with virus proliferation at the level of DNA replication was assumed to be the result of competition for rate-limiting amounts of AC1 protein (Frischmuth & Stanley, 1993). Similarly, this defective interference is also found in transgenic tobacco plants carrying naturally occurring viral subgenomic DNA of BCTV (Frischmuth & Stanley, 1994; Stenger, 1994). However, the protection conferred by the DI interference could not rule out the contribution of additional factors such as the expression of truncated gene(s) from the subgenomic DNA.

Antisense RNA- and Rep protein-mediated resistance. Day et al. (1991) transformed tobacco plants with both sense and antisense AC1 of TGMV. When challenged with TGMV by agroinoculation, only plants expressing antisense RNA conferred a statistical resistance. The resistance was positively correlated with the levels

of the antisense RNA. The viral DNA was not detectable in four out of six lines by leaf disc analysis using DNA A, indicating that the antisense sequences interfered with virus proliferation at the level of DNA replication. The antisense RNA-mediated resistance targeted to the AC1 gene is highly specific, which depends on the level and spacing of homologous sequences between the antisense RNA and its target (Bejarano & Lichtenstein, 1994).

In contrast to the TGMV *AC1* transgenic plants (Day et al., 1991), the ACMV *AC1* transgenic tobacco plants confer virus resistance (Hong & Stanley, 1996). In the latter, five out of seven lines expressed detectable *AC1* mRNA, and showed varied degrees of resistance from asymptomatic to delayed or attenuated symptoms. The resistance is also manifested by significant reduction of viral DNA accumulation in comparison with infected control plants. However, all transgenic plants are susceptible to the related geminiviruses, TGMV and BCTV. The failure to functionally complement the ACMV *AC1* mutant and a lack of detectable protein have led to the question whether the resistance is mediated by the expression of the AC1 protein, by transcription regulation, or both.

Defective MP-mediated resistance. In natural double infections, a plant virus can be complemented by another, frequently unrelated, virus in terms of movement function (Malyshenko et al., 1989). The apparent lack of specificity for the movement function is manifested as the movement protein from a tobamovirus supports the movement requirements of a bromovirus (De Jong & Ahlquist, 1992). Interfering with a function shared by the different viral movement proteins may inhibit the spread of different viruses

(Cooper et al., 1995). This hypothesis has been proved in transgenic plants expressing dysfunctional MPs (Malyshenko et al., 1993; Lapidot et al., 1993; Beck et al., 1994; Cooper et al., 1995). In cases of geminiviruses, von Arnim and Stanley (1992b) demonstrated the inhibition of ACMV infection by co-expression of the TGMV *BC1* gene or a chimeric *BL1* gene. The chimera contains the amino-terminal sequence of the BC1 and a carboxy-terminal sequence of its ACMV homologue BC1. Several different genes at both orientations were individually constructed into the ACMV genome by replacing the CP region. When these recombinants with ACMV were coinoculated, the chimera functioned as a negative dominant mutant, which inhibited the systemic spread of the virus. No inhibition or symptom attenuation was observed in the constructs containing ACMV *BV1*, TGMV *BR1* or the *BC1* and their antisense orientation. Based on these results, von Arnim and Stanley (1992b) suggested the dMP may provide a novel source for geminivirus resistance.

Tomato mottle virus is an important pathogen of tomato in Florida. The virus caused approximately $125 million losses of tomato production in Florida in 1990-1991 (Polston et al., 1993). Currently, the control of TMoV relies on frequent applications of insecticides including imidacloprid, a systemic insecticide because no tolerant or resistant cultivars are available in Florida (J. Polston, personal communication). An alternative way to control TMoV and other geminiviruses would be the development of transgenic plants with viral sequences.

The objectives of this study were to (i) study the expression and function of the nonstructural genes of TMoV in infected and transgenic plants, and (ii) develop transgenic plants resistant to the virus. The nonstructural genes *AC2, AC3, AC4, BC1 and BV1* of TMoV were cloned, and were expressed in *Escherichia coli*. Polyclonal antiserum against each of the expressed proteins AC3, AC4, BC1 and BV1 was produced and was used to study the expression of these nonstructural genes in plants (Duan et al., 1995a). Transgenic plants were generated by *Agrobacterium*-mediated transformation with the *AC4, BC1* and *BV1* gene constructs in sense and/or antisense orientations (Duan et al., 1995b). The phenotype (disease-like symptoms) variations in the transgenic plants expressing the BC1 protein were analyzed. Three spontaneous mutants of the *BC1* transgene were identified from transgenic plants (Duan et al., 1996a). The transgenic plants were evaluated for resistance to several viruses including TMoV, cabbage leaf curl virus (CabLCV), TMV, CMV, TEV and potato virus Y. Transgenic tobacco plants expressing the BC1 mutants were found to be resistant to TMoV and CabLCV (Duan et al., 1996b).

# CHAPTER 2
# SEROLOGICAL DETECTION OF THE NONSTRUCTURAL PROTEINS OF TOMATO MOTTLE VIRUS

## Introduction

Tomato mottle virus (TMoV), a typical bipartite, whitefly-transmitted geminivirus, is an important pathogen of tomato in Florida. Like other bipartite geminiviruses, the TMoV genome is composed of two circular, single-stranded DNA molecules that together contain at least seven open frames (ORFs) (Abouzid et al., 1992b). The A component putatively codes for five proteins, i.e. AC1, AC2, AC3, AC4 and AV1, while the B component codes for two proteins, BC1 and BV1. The homology between the ORFs of TMoV and those of other bipartite geminiviruses varies from 60% to 95% (Abouzid et al., 1992b). The expression of the nonstructural proteins of TMoV in infected plants remains to be investigated.

Some of these proteins in other bipartite geminiviruses such as tomato golden mosaic virus (TGMV), African cassava mosaic virus (ACMV), and squash leaf curl virus (SqLCV), have been detected in infected plants, and their functions have been well characterized. The AC1 protein, essential for DNA replication, has multiple functions such as kinase, endonuclease and helicase (Fontes et al., 1992, 1994; Thommes et al., 1993; Desbiez et al., 1995; Laufs et al., 1995a, 1995b; Orozco & Hanley-Bowdoin, 1995). The AC2 protein facilitates the expression of both the *AV1* and *BV1* genes at the

transcriptional levels of TGMV, serving as a transactivator to activate the promoters of both genes (Sunter & Bisaro, 1992). The AC3 protein is required for efficient DNA accumulation and infection by enhancing viral replication (Elmer et al., 1988; Sunter et al., 1990). The expression of the AC4 ORF has a suppressive effect on expression of the *AC1* gene of TGMV in *Nicotiana benthamiana* protoplasts (Groning et al., 1994). However, mutational analyses indicate that the AC4 ORFs of TGMV and potato yellow mosaic virus do not affect virus replication or symptom development (Elmer et al., 1988; Sung & Coutts, 1995). Furthermore, transient expression of the *AC4* does not affect the *AC1* expression of ACMV in *N. tabacum* protoplasts (Hong & Stanley, 1995). The AC4 protein has only been detected in *in vitro* translation products (Thommes & Buck, 1994). The AV1, the coat protein (CP), is essential for viral encapsidation, and may be required for the systemic infection of bipartite geminiviruses when these viruses infect poorly adapted hosts (Pooma et al., 1996). Two proteins, BC1 and BV1, encoded by the DNA B of bipartite geminiviruses are required for viral infectivity and systemic infection (Brough et al., 1988; Etessami et al., 1988; Von Arnim & Stanley, 1992b; Smith & Maxwell, 1994; Haley et al., 1995; Ingham et al., 1995; Sung & Coutts, 1995). In this study, the TMoV nonstructural proteins were expressed in *E. coli*. Polyclonal antisera raised against each of these expressed proteins were used for the detection of these proteins both in infected and transgenic plants. The AC3, AC4, BC1 and BV1 proteins were detected in transgenic and/or TMoV-infected plants.

## Materials and Methods

### Cloning of AC2, AC3, AC4, BC1 and BV1 ORFs of TMoV

The sequences of AC2, AC3, AC4, BC1 and BV1 ORFs were increased by PCR amplification from a TMoV clone or infected plant extracts based on the nucleotide (nt) sequence of TMoV (Abouzid et al., 1992a). Specific primers were made for each of the AC2 AC3, AC4, BC1 and BV1 ORFs of TMoV, which are 1) 5'-CTTCATATGCGATC TTCATCAAA-3' and 5'-GGTACGTACGCTCGAGCTAGCTAAATAAATTGATCC-3' for AC2; 2) 5'-AAGCTTCATATGGATTCACGCACAGG-3' and 5'-CCTACCTACGC TCGAGTTAATAAAATTTGAATTTT-3' for AC3; 3) 5'-AAGCTTCATATGAAAATG GGGAAC-3' and 5'-TTAATGCTTTCGCATATG-3' for AC4; 4) 5'-AAGCTTATGGA TTCTCA GTTAGT-3' and 5'-GAATTCTTATTTTAATGATTTCGTCT-3' for BC1; and 5) 5'-AAGCTTATGTATCCTTTAAAGAG-3' and 5'-GAATTCTTAACCAATATAATC AAG-3' for BV1. For convenient cloning, the restriction sites either NdeI and EcoR I or Hind III and EcoR I were inserted at each end of the primers for AL2 and AC3, or AC4, BL1 and BR1, respectively, to provide for directional cloning into the pETh-3 vector. The PCR reaction was standardized as the following: 5 µl of the extracts in 100 µl of a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 2.5 mM MgCl, 2.0 mM dNTPs, and 2-5 U Taq polymerase. The thermocycle program was 3 cycles of 94° C for 2 min, 45 ° C for 30 sec, 72 ° C for 1 min; 35 cycles of 92 ° C for 1 min, 50 ° C for 30 sec and 72 ° C for 1 min, and a last cycle of 72 ° C for 5 min in a Biometra UNO-Thermoblock. The PCR products were directly cloned into the pGEM-T

vector (Promega) after gel purification. The DNA fragment of each ORF cloned in the pGEM-T vector was excised by restriction enzymes and subcloned into the correct reading frame of the pETh-3C vector. The pETh-3 vector, selected as the expression vector in the study, is a modification of the original pET-3 vector developed by Studier et al. (1990) for high level expression of genes under the control of the $T_7$ RNA polymerase from bacteriophage $T_7$. All the ORFs except AC3 were constructed to express in *E. coli* strain BL21DE3pLysS as fusion proteins with 11 amino acid (aa) N-terminal peptide (26 aa for AC4) of The $T_7$ gene 10 protein.

Induction and Expression of the Nonstructural Proteins

Plasmid clones containing the AC2, AC3 and AC4 coding sequences, respectively, in the correct orientation and reading frame, were used to transform the appropriate host for expression, *E. coli* strain BL21DE3pLysS. A single transformant colony of each clone grown on LB containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml) was raised in M9 medium (Sambrook et al., 1989) with 0.4% glucose and 0.5% tryptone at 37 C with shaking to an 0.6 $O.D._{600}$. The cultures were induced with 1 mM IPTG, and were allowed to grow an additional 4-5 hr at 37° C. Cells were harvested by centrifugation at 5,000 *g* for 5 min. Pellets were resuspended in one half the original volume of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0), and frozen at -20° C overnight. The cell lysates, after sonication for 5-10 sec, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Antigen Preparation and Antiserum Production

The expressed AC3, AC4, BC1 and BV1 proteins in *E. coli* were partially purified by three cycles of centrifugation at 10,000 *g* for 10 min with washes of the precipitates with TE buffer. The partially purified proteins were separated by preparative SDS-PAGE. The protein bands were visualized by incubating in 0.2 M KCl for 3 min at 4° C. The target protein band was excised and washed three times in cold deionized water, and then frozen at -20° C. The proteins were eluted from the gels using a Bio-Rad electroeluter. The eluted AC3, AC4, BC1 and BV1 proteins were dialyzed overnight against distilled water. Purity of the purified proteins was checked in analytical SDS-PAGE. Thereafter, the proteins were lyophilized.

The antisera to BC1 protein (# 1203) and BV1 protein (# 1205) were prepared as described by Duan et al. (1993) and Wisler et al. (1995) in New Zealand white rabbits by an initial immunization with 2 mg of the expressed protein emulsified in Freund's complete adjuvant, followed by two subsequent intramuscular injections of 2 mg emulsified in Freund's incomplete adjuvant at 2-week and 1-week intervals, respectively, and a final booster injection at an interval of 14 weeks. Rabbits were bled periodically over 6 months. The antisera to AC3 protein (UF 72) and AC4 protein (UF 75) were made by Cocalico Biologicals, Inc. with the following procedure: initial injection of 1.0 mg of antigen, followed by injection of 0.5 mg antigen at 2 weeks, 4 weeks and 5 weeks later. Rabbits were bled twice for test of their titer. The sensitivity and specificity of all antisera were tested by Western blots.

Protein Extraction from Plant Tissue

The subcellular P1, P30 and S30 fractions of leaf tissue extracts from infected and healthy tomato plants (*Lycopersicon esculentum* var. *esculentum* 'Sunny') were prepared as described by Deom et al. (1990) and Pascal et al. (1993). Leaves of tomato plants and/or transgenic tobacco plants (see Chapter 3) were powdered after freezing in liquid nitrogen and then extensively ground with a mortar and pestle in two volumes of ice-cold grinding buffer (GB), which consisted of 100 mM Tris-HCl, pH 8.0, 10 mM EDTA and 5 mM dithiothreitol. The total protein homogenate was centrifuged for 10 min at 1000 *g* to produce supernatant S1 and pellet P1. The supernatant was then centrifuged for 30 min at 30,000 *g* to produce supernatant S30 and pellet P30. Pellet P1 was washed once with GB/Triton (GB supplemented with 1% Triton X-100). The S30 fraction was added to an equal volume of sample buffer (60 mM Tris-HCl, pH 8, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue), while the P30 and P1 were resuspended in 0.6 ml and 3.0 ml sample buffer, respectively. All samples were boiled for 5 min. For the detection of AC3 and AC4 proteins, the fractions were further clarified by acetone precipitation as described by Pedersen and Hanley-Bowdoin (1994).

Twenty grams of healthy leaf tissue were homogenized and centrifuged as described above to produce S1 and P1 fractions. The total protein was concentrated from the S1 fraction by acetone precipitation. The pellet was resuspended in 3 ml GB buffer containing 2.3 % SDS. After boiling for 5 min, the total protein extract was dialyzed overnight against distilled water. The partially purified proteins were added to blocking solution (0.2% I-block, TROPIX, Inc.), in which nitrocellulose membranes were soaked

2-3 hr at room temperature. The membrane was washed once with PBS buffer, then used for preabsorption of the antiserum at room temperature for 1 hr or at 4° C overnight.

Western Blot Analysis

Eight to ten microliters of each fraction were loaded for gel analysis of each sample, except where otherwise noted. The proteins were separated by SDS-PAGE with a modification of the method described by Schagger and von Jagow (1987). The modified stacking gel was 4.5% polyacrylamide (based on the Laemmli system by Towbin et al., 1979). The running gel was prepared with 10% or 12.5% polyacrylamide. The blotting procedure was conducted essentially as described by Towbin et al. (1979) using a Bio-Rad Mini-Protein Electrophoresis Cell and Bio-Rad Trans-Blot Electrophoretic Transfer Cell. The detections of the nonstructural proteins in the extracts from infected and transgenic plants were conducted with Western-Light™ Chemiluminescent Detection System (TROPIX, Inc.) in 1:1000-3000 dilutions of each polyclonal antiserum, which were preabsorbed with partially purified plant proteins.

## Results

Subcloning and Expression of the TMoV Nonstructural Genes in *E. coli*

PCR products of the *AC2, AC3* and *AC4*, *BC1* and *BV1* genes of TMoV were estimated as 450, 400 and 270, 850 and 780 nt, respectively (Fig. 2-1), which agreed with the sizes of TMoV sequence data (Abouzid et al., 1992b). These PCR products were also verified by restriction enzyme digestion (data not shown).

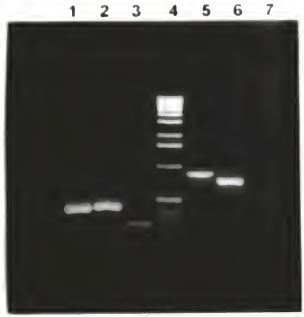



Fig.2-1 Agarose gel electrophoresis of PCR products of the TMoV nonstructural genes amplified from the extracts of TMoV-infected plants.
Lanes 1 to 3, AC2, AC3 and AC4, respectively; Lane 4, 1 kb DNA ladder; Lanes 5 and 6, BC1 and BV1, respectively; Lane 7, healthy tissue extracts as a control.

The induction of the *E. coli* containing the expression vectors with AC3, AC4, BC1 and BV1 ORFs resulted in significant production of each of the AC3, AC4, BC1 and BV1 fusion or non-fusion proteins, respectively. The expression of AC2 ORF in *E. coli*, however, was not high enough for normal isolation, which may be due to the toxicity of the AC2 protein to the bacterium. The expressed proteins with estimated sizes of 16 kDa for AC3, 14 kDa for AC4, 35 kDa for BC1 and 31 kDa for BV1, were insoluble and therefore were readily concentrated from lysed host cells by centrifugation. Each of these expressed proteins was further purified by SDS-PAGE (Fig. 2-2). The polyclonal antiserum raised against each of the expressed proteins AC3, AC4, BC1 and BV1 reacted with its corresponding purified immunogen in Western blot analysis to antiserum dilution of at least at 1:3000.

Detection of the Nonstructural Proteins by Western Blots

The BC1 MP was detected both in the membrane fraction (P30) and cell wall fraction (P1) of infected and transgenic plants but not in extracts of healthy control plants by the preabsorbed anti-BC1 antiserum at 1:3000 dilution. Besides the estimated 33 kDa protein that agreed with the size predicted from the sequence data (Abouzid et al., 1992b), another larger protein (~37 kDa) was also present in the extracts of transgenic tobacco plants (Fig. 2-3, a and b). This phenomenon is consistent with the observation of the BC1 protein detected from ACMV- or SqLCV-infected plants by Western blot analyses (von Arnim et al., 1993; Pascal et al., 1993). The anti-BV1 antiserum raised against the expressed BV1 protein detected a discrete protein of an estimated 29 kDa in both the

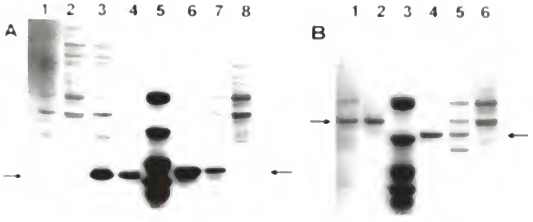



Fig. 2-2. Analysis of the AC3, AC4, BC1 and BV1 proteins expressed in *E. coli*.
Proteins were separated by SDS-PAGE and stained with Coomassie blue. Arrows indicate the expressed nonstructural proteins.
A) Lane 1, induced cell culture with pETh3C vector alone; Lanes 2 and 3, non-induced and induced cell cultures with pETh3C-AC4 clone, respectively; Lane 4, purified AC4 fusion protein; Lane 5, pre-stained low range protein marker (BRL); Lane 6, purified AC3 protein; Lanes 7 and 8, induced and non-induced cell cultures with pETh3C-AC3 clone, respectively.
B) Lane 1, induced culture with pETh3C- BC1 clone; Lane 2, purified BC1 fusion protein; Lane 3, pre-stained low range protein marker; Lane 4, purified BV1 fusion protein; Lanes 5 and 6, induced and non-induced cell cultures with pETh3C-BV1 clone, respectively.

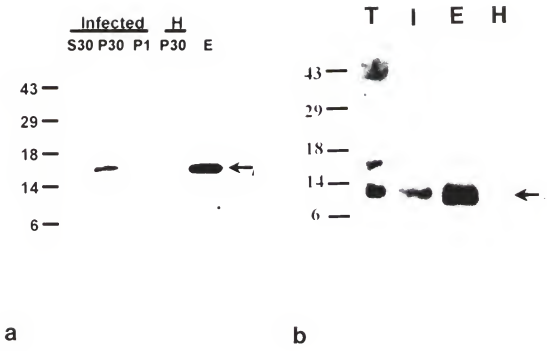



Fig. 2-3. Western blot analysis of the TMoV BC1 protein.
The cell fractions of the leaf tissue extracts from TMoV-infected tomato plants (a) and transgenic tobacco plants (b) were electrophoresed in a 10 % sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, proteins were blotted onto a nitrocellulose membrane, and were detected with the anti-BC1 antiserum at 1:3000 dilution using Western-Light™ Chemiluminescent Detection System. The positions of marker proteins (kDa) are indicated.

soluble fraction (S30) and the membrane fraction (P30) of leaf extracts from TMoV-infected tomato plants, indicating that the antiserum specifically recognized the BV1 protein (Fig. 2-4). In contrast to the detection of BC1 protein in BC1 transgenic plants, the BV1 protein in the BV1 transgenic plants was not readily detected (data not shown). In addition, both the BC1- and BV1-MPs could be detected from the extracts of older leaf tissues of TMoV-infected tomato plants, and the BV1 MP appeared to accumulate as the leaves aged (Fig. 2-5). The anti-AC3 antiserum raised against the expressed AC3 protein detected an estimated 16 kDa protein in the membrane fraction of infected tomato plants, which agreed with the size of AC3 predicted from the sequence data (Fig. 2-6a ). The anti-AC4 antiserum detected an estimated 10-14 kDa protein in the membrane fraction of both infected tomato and transgenic tobacco plants but not in the extracts of healthy control plants, which was in the size range of the AC4 predicted from the sequence data (Fig. 2- 6b).

## Discussion

The TMoV nonstructural proteins, AC3, AC4, BC1, and BV1 were detected in the extracts from TMoV-infected plants and/or transgenic plants by Western blot analysis using the antiserum prepared to each of these proteins expressed in *E. coli*. These results are similar to those reported for TGMV, SqLCV and ACMV (Von Arnim et al., 1993, Pascal et al., 1993; Pedersen & Hanley-Bowdoin, 1994). However, It is the first time that the AC4 protein has been detected in tomato plants infected with a bipartite geminivirus.

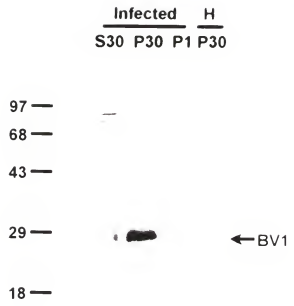



Fig. 2-4. Western blot analysis of the TMoV BV1 protein.
The cell fractions of the leaf tissue extracts from infected tomato plants were electrophoresed in a 10 % sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, proteins were blotted onto a nitrocellulose membrane, and were detected with the anti-BV1 antiserum at 1:3000 dilution using Western-Light™ Chemiluminescent Detection System. The positions of marker proteins (kDa) are indicated.

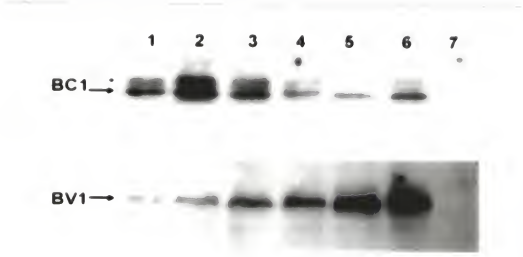



Fig. 2-5. Comparison of the BC1 and BV1 movement proteins in TMoV-infected plants. P30 fractions of the tissue extracts from infected and healthy tomato plants were analyzed by Western blots with the anti-BC1 antiserum and anti-BV1 antiserum, respectively. Lanes 1 to 6, samples from the uppermost expanded leaf (Lane 1) to the sixth leaf down (Lane 6) of TMoV-infected tomato plants one month after inoculation; Lane 7, sample from healthy tomato plants. Each lane was loaded with a sample corresponding to approximately 20 mg of leaf tissue.

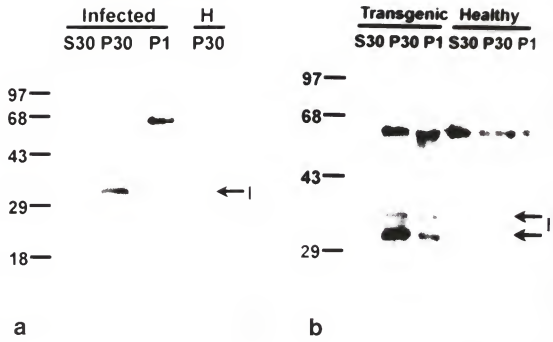



Fig. 2-6. Western blot analysis of the TMoV AC3 and AC4 proteins. The cell fractions of the leaf tissue extracts from transgenic tobacco plants and/ or infected tomato plants were electrophoresed in a 12.5 % sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, proteins were blotted onto a nitrocellulose membrane, and were detected with the anti-AC3 antiserum (a) and the anti-AC4 antiserum at 1:1000 dilution using Western-Light™ Chemiluminescent Detection System. In Fig. 6b, P30 fractions were derived from transgenic healthy (T), TMoV-infected (I) and healthy (H) plant. E, the expressed AC4 protein. The positions of marker proteins are indicated.

A larger protein band besides the predicted 33 kDa protein band of the TMoV BC1 was also detected with the anti-BC1 antiserum in the infected and transgenic plants by Western blot analysis. This phenomenon is similar to those observed in SqLCV- and ACMV-infected plants with Western blot analyses by Von Arnim et al. (1993) and Pascal et al. (1993), who suggested that the larger protein band recognized by the anti-BC1 antiserum in SDS-PAGE may result from post-translational modification of the BC1 protein in infected plants. By labeling studies with $^{32}$P-orthophosphate, $^{3}$H-fucose, or $^{3}$H-glucosamine, Pascal et al. (1994) demonstrated that both BC1 and BV1 expressed in Sf9 cell are phosphorylated but not glycosylated. However, the BV1 proteins of TMoV, SqLCV and ACMV detected by Western blot do not show the anomalous behavior of the BC1 (Figs. 2-4 and 2-5; Von Arnim et al.,1993; Pascal et al.,1993), suggesting that other modifications may occur in the BC1 protein.

The TMoV BV1 protein was primarily detected in the membrane fraction of the tissue extracts from the infected tomato, and this protein appeared to accumulate as leaves aged in contrast to the BC1 protein (Figs. 2-4 and 2-5). Von Arnim et al. (1993) indicated that their BV1 antiserum reacted with both the BV1 and coat protein (CP) of ACMV. However, the antiserum they raised was against a synthetic peptide consisting of 12 amino acids. The anti-BV1 antiserum for ToMV raised to the full-length bacterium-expressed protein was the same as the anti-BV1 antiserum for SqLCV, which specifically recognizes the SqLCV BV1 (Pascal et al., 1993). Since the BV1 protein has been demonstrated to be a nucleic acid binding and a nuclear shuttle protein (Sanderfoot &

Lazarowitz, 1996), the accumulation of the protein as leaves aged may imply that this protein may accumulate in the nuclei.

A number of reports on site-directed mutagenesis studies of the *AC4* gene have concluded that this gene is not necessary for bipartite geminivirus replication (Elmer et al., 1988; Hong & Stanley1995; Sung & Coutts, 1995). These studies have not tested whether repair mechanisms have restored the mutated sites in *AC4* during viral replication by using serology to detect expressed AC4 protein. *In vitro*, the TGMV AC4 protein was synthesized in a large excess over the AC1 protein (Thommes & Buck, 1994). The detection of TMoV AC4 protein in infected tomato (Fig. 2-6) implies that this gene may have a role in TMoV replication. Preliminary tests with transgenic tobacco carrying the *AC4* gene (see Chapter 3) show resistance to TMoV infection (data not shown).

# CHAPTER 3
# PHENOTYPIC VARIATION IN TRANSGENIC TOBACCO EXPRESSING MUTATED GEMINIVIRUS MOVEMENT/PATHOGENICITY (BC1) PROTEINS

## Introduction

*Agrobacterium*-mediated transformation is a widely used method for inserting genes into plant genomes for both basic and applied studies (Martineau et al., 1994). Transformation of plants with viral sequences may result in plants with virus resistance (Beachy, 1993). This phenomenon is known as "pathogen-derived resistance" (Sanford and Johnson, 1985). The level of resistance obtained is variable (Hull, 1994), and this variability has been attributed to the random nature of the transformation process (Lomonossoff, 1995). Independent lines of plants generated from a single transformation experiment may contain different transgene copy numbers with insertion in various chromosomes. Phenotypic differences are also noted among plant lines containing a single copy of the transgene. Some of the variability in transgene expression has also been attributed to tissue culture-induced changes (Phillips et al., 1994). This variability in the phenotype is also observed in subsequent progeny derived from the $T_0$ plants.

Transgenic plants expressing viral movement protein (MP) have provided valuable information regarding MP biochemical functions and in defining MP contributions to viral infection (Cooper et al., 1995; Lapidot et al., 1993). Plant virus MPs have also been studied for their potential utilization as novel sources for virus resistance genes (Von

Arnim and Stanley, 1992b). For DNA bipartite geminiviruses, the *BC1* (also known as BL1 in the literature) gene has been identified as a symptom-inducing element with virus movement function (von Arnim and Stanley, 1992a; Pascal et al., 1993; Noueiry et al., 1994; Pascal et al., 1994; Smith and Maxwell, 1994; Ingham et al., 1995). Von Arnim and Stanley (1992a) constructed pseudorecombinants between the common and the yellow strains of tomato golden mosaic virus (TGMV), produced by exchange of genomic components between the strains, and mapped the determinants of symptom development to the *BC1* gene. Pascal et al. (1993) reported that the expression of squash leaf curl geminivirus (SqLCV) BC1 MP in transgenic tobacco was sufficient to produce symptoms typical of SqLCV infection. The BC1 protein localizes to the cell wall and plasma membrane (Pascal et al., 1993) and increases the size exclusion limit of the plasmodesmal pores in the cell walls (Noueiry et al., 1994). Based on these studies and in comparison with MPs of RNA plant viruses, the BC1 protein is predicted to function in the cell-to-cell and long distance movement of bipartite geminiviruses (Pascal et al., 1993, 1994; Noueiry et al., 1994).

In this study, transgenic tobacco plants were generated by *Agrobacterium*-mediated transformation with the TMoV *AC4*, *BC1* and *BV1* gene constructs in antisense and/or sense orientations for evaluation of gene function(s) and for possible utilization in pathogen-derived resistance. Transgenic plants expressing the *BC1* gene showed a range of phenotypes from severe stunting and leaf mottling to no visible symptoms. The variations in the phenotype were found to be associated with transgene mutations.

## Materials and Methods

### Clone Construction and Plant Transformation.

The DNA fragments for *AC4, BC1* and *BV1* genes of tomato mottle geminivirus (TMoV) (Abouzid et al., 1992a) were amplified from the extracts of TMoV infected tomato plants by polymerase chain reaction (PCR). The primers for *AC4*, *BC1* and *BV1* were 1) TGAAAGCTTATCCCCAGTGCTCTC-3' and 5'-TGACTCGAGGCTTGCCTG TGTGCTC-3'; 2) 5'-CCCAACTTCGAGTTCGAAACTGC-3' and 5'-CCCAAGCTTAAC GAAGTGTGTTTGAC-3'; and 3) 5'-CCCAAGCTTTATCCAACTCAGCTGCA-3' and 5-'CCCAAGCTTGTAAGGCGTGTCAGAC-3', respectively. The amplified *AC4* [nucleotides (nts) between 2259 and 1552], *BC1* (nts between 1278 and 2308) and *BV1* (nts between 500 and 1200) segments were cloned into the pGEM-T vector and then digested with Hind III and XbaI for *AC4*, and with Hind III for *BC1* and *BV1*. The excised *AC4*, *BC1* and *BV1* segments were ligated into the unique Hind III site (*BC1* and *BV1*) and XbaI and HindIII sites (*AC4*) of the binary vector, pKYLX 71:35 $S^2$ (Maiti et al., 1993) to create the sense clones pKYsAC4, pKYsBC1 and pKYsBV1 and the antisense clones pKYasBC1 and pKYasBV1 (Fig. 3-1). Each of these clones was transformed into cells of *Agrobacterium tumefaciens* LBA 4404 (An et al., 1985). *Nicotiana tabacum* cv. Xanthi was transformed using the leaf disc method with MS selective medium containing cefotaxin 250 μg/ml and 100 μg/ml kanamycin (Horsch et al., 1985). Transformants were identified by PCR using specific primers, by ELISA tests using an NPT II (neomycin phosphotransferase II) ELISA Kit (5 Prime→3 Prime, Inc.).

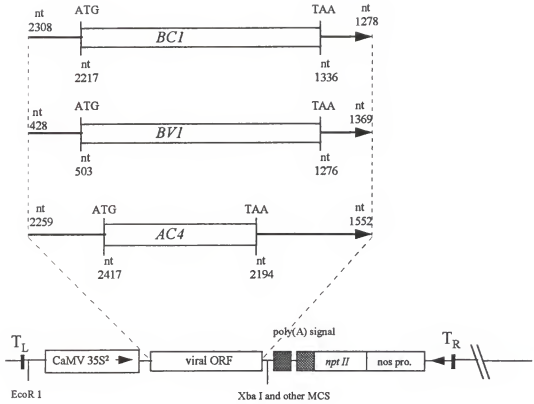



Fig. 3-1. Construction of expression vector for the TMoV nonstructural protein genes with the plasmid pKYLX71:35S$^2$.

The *AC4*, *BC1* and *BV1* ORFs including their 5'-leading and 3'-nontranslated intergenic sequences were amplified by PCR using primers with compatible restriction endonuclease site(s). Each of these PCR products was cloned into the pGEM-T vector. The BC1 and BV1 clones were digested with Hind III, while the AC4 clone was digested with Hind III and XhoI.

Each of the genes was ligated into the pKYLX71:35S$^2$ vector to generate both sense and antisense of *BC1*- and *BV1*-pKYLX71:35S$^2$ and only sense *AC4*- pKYLX71:35S$^2$.

The positions of translational start and termination codons are indicated. The left and right T-DNA border sequences of the expression vectors are represented by $T_L$ and $T_R$. The cauliflower mosaic virus 35S promoter, nos promoter (nos pro.), neomycin phosphotransferase II gene (*npt II*), polyadenylation signal (poly (A) signal), multiple cloning sites (MCS) and nucleotide (nt) are noted.

and by Southern blot analysis.The $T_0$ plants were self-pollinated to produce the $T_1$ generation, and some selected lines were selfed to obtained the $T_2$ generation. The $T_1$ and $T_2$ seeds of selected lines were germinated individually on MS medium containing 100 μg/ml kanamycin. The $T_1$ and $T_2$ seedlings were transplanted into soil a month after germination.

DNA and RNA Extraction

DNA was isolated by a modification of the method of Cocciolone and Cone (1993). Young tobacco leaf tissue (0.2 g) was ground in liquid nitrogen with 1.0 ml extraction buffer (7 M urea, 0.35 M NaCl, 50 mM Tris pH 8.0, 20 mM EDTA, 1% sarkosyl) in a mortar and pestle. This mixture was transferred to a 2 ml micro-centrifuge tube, and was extracted with an equal volume of a solution containing phenol: chloroform-isoamyl alcohol (25:24:1). After centrifugation at 3000*g* for 5 min, the supernatant was extracted with chloroform-isoamyl alcohol (24:1), and was centrifuged at 6000*g* for 5 min. An equal volume of isopropanol was added to the supernatant and the mixture was stored at -20° C for one hr. The nucleic acid was precipitated by centrifugation at 6000*g* for 20 min, and resuspended in 0.4 ml TE-SAR (10 mM Tris pH 8.0, 1 mM EDTA, 0.5% sarkosyl) with 10 μg RNase. After incubation at 37° C for 30 min, the mixture was extracted once with phenol:chloroform and then with chloroform, and was re-precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes 100% ethanol. DNA samples were resuspended in TE and stored at -20° C.

RNA was isolated by a modification of the DNA isolation method described by Cocciolone and Cone (1993). Samples were prepared and extracted as above until just before the isopropanol addition. After the addition of an equal volume of isopropanol to the supernatant and chilling on ice for 5 min, a heavy precipitate formed. The soluble fraction was removed from the heavy precipitation with a pipette. The soluble fraction containing a large amount of RNA and a trace amount of DNA was precipitated by centrifugation at 16,000*g* for 10 min after incubation at -20° C for one hr. The pellet was washed with 70% ethanol, and resuspended in DEPC-$H_2O$ for direct use, or resuspended in 0.4 ml TE-SAR, and extracted once with phenol:chloroform, and stored at -20° C after the addition of 0.1 volume of 3 M sodium acetate and 2 volumes of 100% ethanol.

Southern and Northern Blot Analyses

Ten μg of genomic DNA were digested 3-6 hr with restriction enzymes (EcoR I or XbaI, both of which have a unique site within the vector T-DNA; Maiti et al., 1993). The digested DNA was separated by electrophoresis on an 0.75% agarose gel and transferred to nylon membrane with alkaline transfer buffer. Probe was made by random primed $^{32}$P-labeling of the TMoV BC1 DNA fragment (nucleotides 1278-2308) with a Prime-a Gene labeling kit (Promega Co., Madison, WI). Hybridization (overnight at 65° C) of blots was in 6 x SSC, 5 x Denhardt's solution and 0.5% SDS, 0.1 mg /ml salmon sperm DNA. Blots were washed twice in 2 x SSC, 0.1% SDS at room temperature for 10 min, and once in 1 x SSC, 0.1% SDS and in 0.1 x SSC, 0.1% SDS at 65° C for 15 min (Sambrook et al., 1989).

Ten to fifteen μg of total RNA were separated on a 1.0% agarose gel with formaldehyde, and blotted onto nylon membranes. Hybridization was carried out as described above for DNA gel blots.

Western Blot Analysis

The subcellular fractions of the leaf extracts from transgenic and control (both TMoV-infected and non-infected tobacco) plants were prepared as described by Pascal et al. (1993). Eight microliters of each sample (P30 fraction) were loaded for gel analysis. The proteins were separated by SDS-PAGE with a modification of the method described by Schagger and von Jagow (1987). The modified stacking gel was 4.5% polyacrylamide (based on the Laemmli system). After electrophoresis, the separated proteins were transferred to nitrocelluose. The BC1 proteins were immuno-reacted with polyclonal antiserum (preabsorbed with partially purified tobacco proteins) against bacterium-expressed BC1 protein (Duan et al., 1995a) and immuno-reactions were detected with Western-Light™ Chemiluminescent Detection System (TROPIX, Inc.).

Sequence Analysis

The *BC1* transgene was amplified by PCR from genomic DNA using specific primers for the TMoV *BC1* gene (EH 262, 5'-CCCAAGCTTCGAGTTCGAAACTGC-3' and EH 263, 5'-CCCAAGCT TAACGAAGTGTGTTTGAC-3') and for *rbcS* terminator sequence (EH 274, 5'-TCGATTGATGCATGTTGTC-3'). Reactions were run in a thermocycler (Biometra Inc., Tampa, FL) for 30 cycles with a touchdown program

composed of a 2 min denaturation at 92° C, 30 sec annealing time at 60-50° C (-1° C each cycle) and 1 min extension at 72° C, for the first 10 cycles, and a 1 min denaturation at 92° C, 30 sec annealing time at 50° C, 1 min extension 72° C, for the other 20 cycles. RT-PCR was carried out from total RNA using SuperScript™ reverse transcriptase (GIBO BRL) with an oligo(dT) primer and *Taq* polymerase (Promega Co, Madison, WI) with an EH 262 primer for the *BC1*. To confirm the BC1t/r sequence resolved from the cDNA, the transgene sequence was amplified by PCR from genomic DNA using primers EH 262 and EH 278, 5'-GAGCTCAGAAATAATTTGGAAAC-3' designed on the basis of the cDNA sequence at the 3' end. PCR products were directly sequenced, or were cloned into pGEM T-vector (Promega Co, Madison, WI), and then were sequenced. Sequencing was done in both directions and repeated at least once with an independent reaction. DNA samples were sequenced by the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR). The Taq DyeDeoxy Terminator Cycle Sequencing protocols developed by Applied Biosystems with fluorescent-labeled dideoxynucleotides and primers were used. The sequences were analyzed using the University of Florida ICBR Computer Core which has the University of Wisconsin Genetics Computer Group software.

Starch-Iodine Staining

Newly expanded leaves were sampled from TMoV-infected (30 days post-inoculation), transgenic and control tobacco plants in the morning. Leaves were decolorized in 70% ethanol, and stained with iodine-potassium iodide-lactic acid mixture

(stock IKI was 2% iodine and 6% KI, mixed with 85% lactic acid in 1:20 ratio) as described by Lindner et al. (1959).

## Results

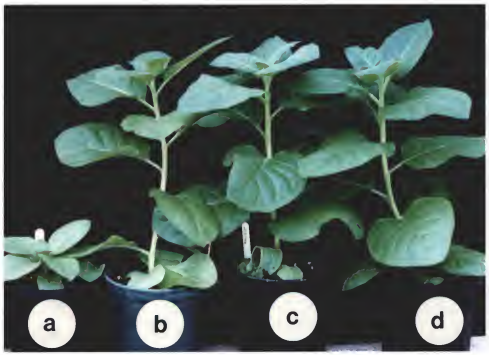
### Unexpected Phenotypic Variation In *BC1* Transgenic Tobacco

A total of 132 tobacco plants were generated from the transformations. Nineteen BC1 sense, 16 BC1 antisense, 20 BV1 sense dimer, 7 BV1 sense, 5 BV1 antisense, and 21 AC4 sense (Fig. 3-1) transgenic plants were obtained. Most of these transgenic plants, except for some of the plants expressing the *BC1* gene, had a phenotype indistinguishable from non-transformed tobacco. However, only 11 of the 19 transgenic $T_0$ tobacco plants that expressed the BC1 protein showed disease symptoms, which ranged from mild mottling to severe stunting and mottling mimic to the disease symptoms induced by TMoV infection (Polston et al., 1993). Eight plants expressing the BC1 protein did not show any apparent symptoms.

A $T_0$ plant (BC3-11) with only mild mottling symptoms was self-pollinated and at least three phenotypes were observed in the $T_1$ generation (Fig. 3-2). These phenotypes were type "S"= severe stunting and mottling, more severe than the typical symptoms associated with TMoV infections in tobacco; type "M" = mild mottling with no stunting of growth; and type "A" = asymptomatic, no visible symptoms, plants indistinguishable from uninfected, non-transformed plants.

Fig. 3-2. Phenotypic comparison of transgenic $T_1$ tobacco plants expressing TMoV BC1 protein.
Transgenic plants were derived from a $T_0$ plant that showed mild leaf mottling with no stunting.
(A) Plants from left to right: a. transgenic plant (BC3-11-5S) showing stunting and leaf mottling, more severe than those induced by TMoV infection; b. transgenic plant (BC3-11-2M), showing mottling with no stunting; c. transgenic plant (BC3-11-6A) with no apparent symptoms; and d. non-transformed tobacco plant.
(B) Plant on the left is representative of the genotype as in b, Fig. 3-2A and on the right as in c Fig. 3-2A. The plants in A and B were photographed 45 days and 90 days after transplanting, respectively.

A




B

Genetic Characterization of Transgenic Lines

Transgenic $T_1$ plants were selected on the basis of phenotype and analyzed by DNA blots to verify the presence and segregation of the transgene and to establish gene copy number (Fig. 3-3). The $T_1$ plants with the "M" phenotype (BC3-11-2M) had two copies of the *BC1* gene. Other $T_1$ progeny from this line which had an "S" phenotype (BC3-11-5S) or an "A" phenotype (BC3-1-6A) only had one copy (Fig. 3-3). For comparative purposes, progeny from three other lines with the "A" phenotype were also examined and found to have 3, 3, and 5 copies of the *BC1* gene, respectively (Fig. 3-3). The transgene copy numbers identified for the transgenic lines were consistent for genomic DNA digested with either XbaI (Fig. 3) or EcoRI (not shown).

Expression of *BC1* in Transgenic Plants

High levels of the BC1 protein expression were indicated in the young tissues of all transgenic plants by protein immunoblot analysis, regardless of the phenotype except for one "A" phenotype plant. Extracts from the latter showed low levels of a smaller (23 kDa versus expected 33 kDa) BC1-related protein (Fig. 3-4). Three plant lines with the "A" phenotype (BC3-31-3A, BC3-6-4A, and BC3-6-3A) had a similar size and level of BC1 protein as the "S" phenotype plant (BC3-11-5S), indicating that the expression level of the BC1 protein apparently was not correlated with the phenotypes (Fig. 3-4). Extracts from the "M" phenotype plant (Fig. 3-2; Fig. 3-4, BC3-11-2M) showed the expected BC1 protein and a trace amount of a smaller, 23 kDa BC1-related protein. It was difficult to

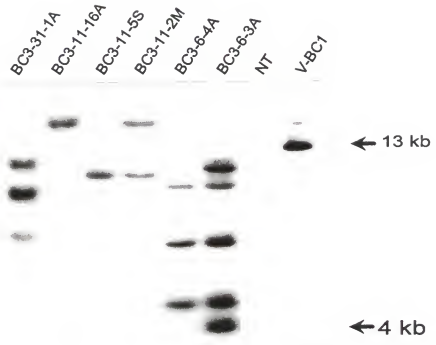



Fig. 3-3. Southern blot analysis of the $T_1$ transgenic plants with different phenotypes. Segregation of the *BC1* transgene in $T_1$ generation of transgenic tobacco plants which displayed different phenotypes in Figure 2. Blots for BC3-31-1A, BC3-6-4A, and B3-6-3A plant lines with no visible symptom phenotype are shown for comparative purposes; NT = nontransformed plant; and V-*BC1* = pKYLX71:35S[2] vector with *BC1* ORF construct used for transformation. Genomic DNA of the transgenic plants was extracted and digested with Xba I. DNA blots were subjected to hybridization with $^{32}$P-labeled *BC1* DNA fragment. BC3-11-6A, BC3-11-5S, and BC3-11-2M were derived from a $T_0$ line BC3-11 (two copies, data not shown), BC3-6-4A and BC3-6-3A were derived from $T_0$ line BC3-6 (asymptomatic phenotype; 5 copies, data not shown) and BC3-31-1A was derived from $T_0$ line BC3-31 (asymptomatic phenotype; 4 copies, data not shown).

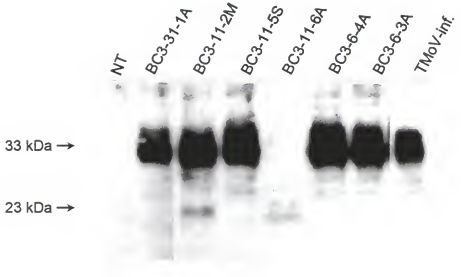



Fig. 3-4. Western blot analysis of the P30 fraction of tissue extracts from transgenic $T_1$ tobacco plants expressing the *BC1* gene.
Young leaf tissue extracts were fractionated into S30 (soluble fraction), P30 (membrane fraction) and P1(cell wall fraction). Eight microliters of the P30 fractions from transgenic lines were electrophoresed on SDS-PAGE and transferred to nitrocellulose membrane. The BC1 protein was detected with the anti-BC1 antiserum at 1:3000 dilution. The estimated size of the full length (33 kDa) and the truncated (23 kDa) BC1 proteins are indicated. Lanes represent extracts from plants described in Fig. 3-3 except for TMoV-inf. (extract from TMoV infected tissue).

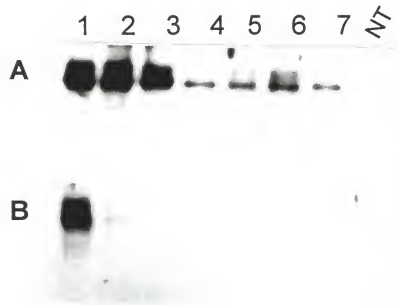



Fig. 3-5. Western blot analysis of the stability of the BC1 protein in transgenic tobacco plants.

Leaves were sampled from the youngest leaf to the oldest leaf of transgenic lines at 76 days after transplanting in the greenhouse. Tissue extracts were from similar leaf positions for (**A**) and (**B**). Lanes 1-7 represent the BC1 protein detection in extracts ( P30 fraction) from the youngest leaf to the oldest leaf with every 3 leaf interval, and NT = young leaf tissue extract from nontransformed tobacco plant. Eight microliters of each sample were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with the polyclonal antiserum as in Fig. 3-4.

(**A**) Western blot analysis of the leaf tissue extracts from a symptomatic plant (BC3-11-5S).

(**B**) Western blot analysis of the leaf tissue extracts from an asymptomatic plant (BC3-31-1A).

detect the BC1 protein in older leaves of the "A" phenotype plants. The stability of the BC1 protein in various leaves from a "S" phenotype plant (BC3-11-5S) was compared with that from an "A" phenotype plant (BC3-31-1A) by sampling every third leaf starting from the top down in 3 month old transgenic plants. Instability of the BC1 protein in an "A" phenotype was indicated by the detection of very low protein levels in extracts from older leaves compared to levels in the "S" phenotype extracts (Fig. 3-5). RNA blots indicated a high steady state mRNA level for all the transgenic lines (Fig. 3-6). The "A" phenotype plant shown in Fig. 3-2A had a transcript size of ~ 0.9 kb (BC3-11-6A) versus the expected ~ 1.0 kb transcript seen in the "S" phenotype (BC3-11-5S) or in other "A" phenotype plants (BC3-31-1A, BC3-6-4A, and BC3-6-3A). The expected transcript size assumes that the transcription termination signals of the TMoV BC1 downstream from the open reading frame (ORF) are active (Fig. 3-1). The smaller transcript for BC3-11-6A is consistent with the smaller BC1 related protein seen for the "A" phenotype in Western blots (Fig. 3-4, BC3-11-6A). The "M" phenotype plant contained both the smaller and the expected size transcripts. The transcript level for the "A" phenotype plant expressing the smaller BC1-related protein was high and therefore the low level of BC1-related protein detected in the Western blot (Fig. 3-4) was not related to transcript abundance. The 1.2 kb transcript, which was seen in all extracts except for the one with the smaller transcript (Fig. 3-6), was the result of a readthrough of *BC1* termination signals into the *rbcS* termination sequences of the pKYLX71:35S[2] vector (Maiti et al., 1993) (sequence data not shown). This was determined by sequencing products obtained after reverse transcriptase reaction of total RNA extract using oligo(dT) primer and then PCR

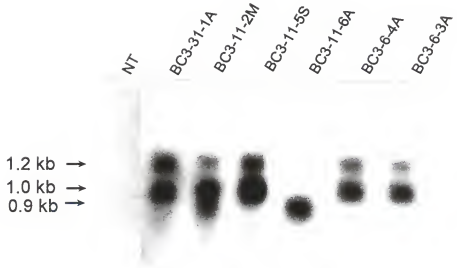



Fig. 3-6. Northern blot analysis of transgenic plants which express the *BC1* gene, probed with labeled-*BC1* DNA.
Total RNA , extracted from transgenic plants and a nontransformed plant, was separated on a denaturing agarose gel with formaldehyde, and then transferred to a nylon membrane. The *BC1* transgene transcripts were detected by probing with $^{32}P$ labeled-*BC1* DNA. The estimated sizes of the two *BC1* related transcripts for full-length *BC1* gene and one transcript for a 3'-truncated/recombination form of the *BC1* gene are indicated. The samples indicated are as in Fig. 3-4.

Fig. 3-7. Nucleotide sequences (A) and predicted amino acid sequences (B) of the TMoV BC1 and its mutated forms.

The nucleotide sequence of the TMoV *BC1* gene from GenBank Accession U14461. The sequence of the PCR amplified *BC1* ORF was verified before and after cloning into pGEM-T vector. The *BC1A* sequence determined from an asymptomatic, multicopy transgenic plant which expressed full length BC1 protein. The sequence was analyzed from the PCR product derived from genomic DNA (BC3-6-3A). The *BC1*At/r sequence determined from the cDNA, the RT-PCR products, amplified from the total RNA (BC3-11-6A). The sequence was also verified by sequencing the PCR product from the genomic DNA and from cloned PCR product. The *BC1S* sequence determined from a symptomatic transgenic plant which expressed full length BC1 protein. The sequence was analyzed after RT-PCR of total RNA (BC3-11-5S), after PCR amplification of genomic DNA (BC3-11-5S) and after PCR amplification from 3 different lines with a similar phenotype.
Note that identical or different nucleotides and amino acid residues are indicated by (.), or bold letters. Letters with underline represent start or stop codons.

```
            1                                                   50
BC1wt       CGAGTTCGAA ACTGCCGTTT CATAGCTTGT TCGACTTCTT TCTGATTCAA
BC1S        .......... .......... .......... .......... ..........
BC1A        .......... .......... .......... .......... ..........
BC1scDNA    .......... .......... .......... .......... ..........
BC1t/rCDNA  .......... .......... .......... .......... ..........

           51                                                  100
            CTTCAGCAAT CACTGCCGCT GCGCGGCCTT ATTTTCATTA TATGGATTCT
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........

           101                                                  150
            CAGTTAGTTA ATCCTCCTAG TGCATTCAAC TACATAGAGT CACACCGTGA
            .......... .......... .......... .......... ..........
            ......T... G......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........

           151                                                  200
            CGAATATCAG CTTTCTCATG ACCTAACTGA GATAATACTG CAGTTTCCGT
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ......A...
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........

           201                                                  250
            CCACGGCGTC GCAGTTAACC GCTAGGCTCA GCCGTAGCTG CATGAAAATC
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........

           251                                                  300
            GACCACTGCG TCATAGAGTA CAGACAACAA GTACCAATAA ACGCCACTGG
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........

           301                                                  350
            GTCGGTAATA GTGGAGATTC ACGACAAAAG GATGACGGAG AATGAGTCTT
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........

           351                                                  400
            TACAGGCATC ATGGACATTT CCGATCAGGT GCAACATAGA TCTCCACTAT
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
            .......... .......... .......... .......... ..........
```

```
401                                                                 450
TTCTCAGCTT CCTTCTTCTC CTTGAAAGAC CCAATTCCAT GGAAATTGTA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

451                                                                 500
TTACAGGGTT TGCGATACGA ATGTTCATCA ACGGACCCAC TTCGCCAAGT
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

501                                                                 550
TTAAGGGGAA GCTGAAATTG TCCACAGCAA AACACTCAGT AGACATTCCC
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

551                                                                 600
TTCCGGGCAC CAACAGTAAA AATCCTGTCC AAACAGTTCA CAGATAAAGA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

601                                                                 650
TGTGGACTTT TCCCATGTGG ATTACGGTAA ATGGGAGAGG AAGCCCATTA
.......... .......... ....T..... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... ....T..... .......... ..........
TGTGGACTTT TC........ ACCATTGAAA CTGAGCCAAA AATGCATGTT

651                                                                 700
GATGCGCGTC TATGTCCAGA CTTGGGCTTA GAGGCCCAAT TGAGATCAGG
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
TTGGGGGAAA ATTCCAAATA GTGCAACTTT TGAACAAACA TACGATGGCT

701                                                                 750
CCTGGTGAGT CATGGGCTTC AAGGAGTACA ATAGGCATAG GGCATTCAGA
.......... .......... .......... ...A...... ......T...
.......... .......... .......... .......... ..........
.......... .......... .......... ...A...... ......T...
AAAGTAGTAA CAAGGCAAAG GACGTAAACC TAAAATGATG TCCAAAAGCG

751                                                                 800
TGCAGACTCA GAAGTGGAGA ACGAACTCCA CCCGTACAGA CATCTAAACA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
TAATTATTCA CTGTAGATGT TTGTTTTACC AACCAAAGAA AAACTAAATT
```

Fig. 3-7A--continued

```
801                                                             850
GGCTAGGAAC AGGCATACTG GACCCGGGAG AGTCTGCTTC TATTGTGGGG
.......... .......... .......... G......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... G......... ..........
GTGTATCCTA ATCAACAAAC TATCAGTAAG TTTCCAAATT ATTTCTAAAA

851                                                             900
GCCCAGAAAG CAGAGTCCAA CATTACAATG TCTATGGGTC AGTTGAACGA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
AAAAAAAAAA .......... .......... .......... ..........

901                                                             950
ATTAATACGG ACTACGGTCC ATGAATGTAT TAATAGTAAT TGTAAGGCGT
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

951                                                            1000
CTCAGACGAA ATCATTAAAA TAAAATTTTA TTTTTACATT TTCATTTATG
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

1001                            1031
TTAATCATCT TTAGTCAAAC ACACTTCGTT A
.......... .......... .......... .
.......... .......... .......... .
AAAAAAAAAA AAAAA..... .......... .
```

Fig. 3-7A --continued

```
          1                                                   50
BC1wt     MDSQLVNPPS AFNYIESHRD EYQLSHDLTE IILQFPSTAS QLTARLSRSC
BC1A      .....FS.......... .......... ........L.......... .....
BC1S      .......... .......... .......... .......... ..........
BC1At/r   .......... .......... .......... .......... ..........

          51                                                  100
BC1wt     MKIDHCVIEY RQQVPINATG SVIVEIHDKR MTENESLQAS WTFPIRCNID
BC1A      .......... .......... .......... .......... ..........
BC1S      .......... .......... .......... .......... ..........
BC1At/r   .......... .......... .......... .......... ..........

        101                                                   150
BC1wt     LHYFSASFFS LKDPIPWKLY YRVCDTNVHQ RTHFAKFKGK LKLSTAKHSV
BC1A      .......... .......... .......... .......... ..........
BC1S      .......... .......... .......... .......... ..........
BC1At/r   .......... .......... .......... .......... ..........

          151                                                 200
BC1wt     DIPFRAPTVK ILSKQFTDKD VDFSHVDYGK WERKPIRCAS MSRLGLRGPI
BC1A      .......... .......... .......... .......... ..........
BC1S      .......... .......... .......... .......... ..........
BC1At/r   .......... .......... ....PLKLSQ KCMFWGKIPN SATFEQTYDG*

          201                                                 250
BC1wt     EIRPGESWAS RSTIGIGHSD ADSEVENELH PYRHLNRLGT GILDPGESAS
BC1A      .......... .......... .......... .......... ..........
BC1S      .......... ....S...L. .......... .......... ......G...

          251                                           294
BC1wt     IVGAQKAESN ITMSMGQLNE LIRTTVHECI NSNCKASQTK SLK*
BC1A      .......... .......... .......... .......... ...*
BC1S      .......... .......... .......... .......... ...*
```

Fig. 3-7B

amplification with the specific 5' primer for *BC1* and the oligo(dT) primer. One of the products had a 3' termination sequence expected for the *BC1* gene while the larger product had the expected *BC1* gene sequence plus *rbcS* termination sequence.

Transgene Sequence Analysis

The variations in the phenotype in transgenic tobacco lines expressing similar levels of BC1 protein indicated possible mutations in the *BC1* gene. The *BC1* gene from transgenic tobacco plants showing the "S" (BC3-11-5) or "A" (BC3-11-6) phenotype was amplified by polymerase chain reaction (PCR) and sequenced; and the open reading frame (ORF) translated into amino acid residues. The sequence data (for 4 independent lines) revealed mutations (amino acid residues 215 G to S, 219 S to L, and 247 E to G) near the carboxyl terminus of the BC1 protein (Fig. 3-7, *BC1*S) for the "S" phenotype (Figure 2A, plant a). This sequence was identical to that resolved from PCR amplified product from the *Agrobacterium* culture used for transformation (data not shown). The primers used for the construction of the *BC1* gene failed to amplify any product from the single copy "A" phenotype line in PCR reactions. A product representing the *BC1* sequence was amplified using the 5' specific primer and an internal primer, indicating that a partial copy of the *BC1* gene was present in the "A" phenotype line. Several other 3' end primers based on the *rbcS* termination sequences of the pKYLX71:35S[2] vector, also failed. Product suitable for sequencing was obtained after reverse transcriptase reaction of total RNA extract using an oligo(dT) primer and then PCR amplification with the specific 5' primer for *BC1* and the oligo(dT) primer. The first 616 nucleotide bases were identical to

the *BC1*S sequence but the 3' end had 225 unique bases, not including the poly A tail (data not shown). The recombinational addition of the unique 225 bases included 81 in phase with the *BC1* ORF, and 144 bases presumably with transcriptional terminator signals. The amino acid sequence for the "A" phenotype (resolved from BC3-11-6A, Fig. 3-2A; Fig. 3-7, *BC1*At/r) showed a deletion of amino acid residues 174 -293, and an additional sequence of 26 amino acid residues, from an unidentified recombinational origin, starting after amino acid residue 173. The ORF predicted for the *BC1*At/r sequence was consistent with the detection of a smaller BC1 related protein (~10 kDa smaller in size compared to the wild type; Fig.3-4) in the protein immunoblot and a reduced transcript size in the RNA blot (Fig.3-6). The low level of the truncated BC1 protein detection may be due to the loss of epitopes since 121 amino acid residues were lost at the carboxy end of the protein. The sequence for another line with an "A" phenotype (resolved from a clone derived from PCR amplification of line BC3-6-3A; Fig. 3-7, *BC1*A) showed several changes near the amino terminus (amino acid residues 6 V to F, 7 N to S, and 35 F to L).

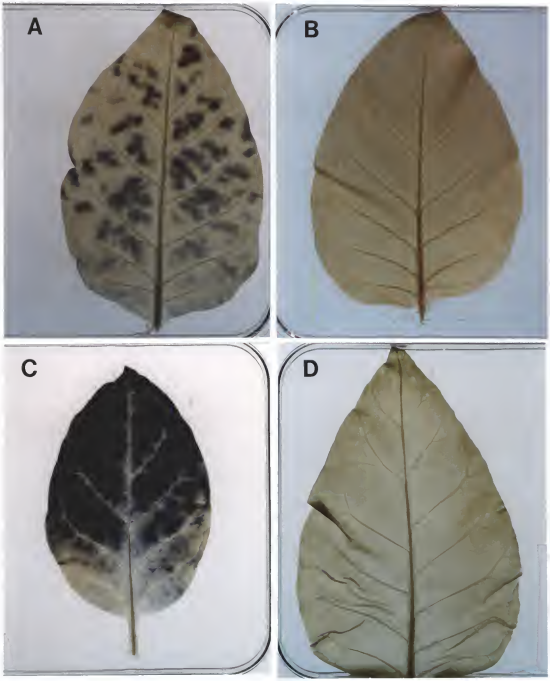
In order to exclude possible PCR artifacts, the sequence analyses were done in several different ways and with independent reactions. For the "S" phenotype line BC3-11-5S and "A" phenotype line BC3-11-6A sequences were obtained with independent reactions by sequencing cloned PCR products and by direct sequencing of PCR products from cDNA and from genomic DNA. Three other lines with the "S" phenotype (including one single copy line and 2 multiple sequence repeat lines, not shown) were also sequenced after PCR amplification of genomic DNA and the sequences were identical to *BC1*S. The

sequence for BC3-6-3A line (multiple sequence repeat line) was resolved from two different clones of PCR product amplified from genomic DNA. The sequence for the *BC1* gene in the pKYLX71:35S$^2$ vector (identical to the *BC1*S sequence) from the *Agrobacterium* culture used for transformation was done once from a PCR amplification of isolated plasmid DNA (data not shown). In the latter case we can not exclude the possibility that other *BC1* gene sequence variations exist in the plasmid population in the *Agrobacterium* culture.

Symptomatic Phenotype Mimics Impaired Photoassimilate Partitioning

One of the processes involved in plant virus disease induction is the reduction in translocation of fixed carbon out of the leaves (Bos, 1970). Starch accumulation in the leaf causes alterations in leaf texture and color. The symptomatic and asymptomatic *BC1* transgenic tobacco as well as TMoV infected tobacco were tested for altered carbon partitioning, determined by the starch-iodine test (Fig. 3-8). Symptomatic plants showed high levels of starch accumulation. Starch accumulation was not detected in the asymptomatic transgenic plants or in control plants by this test. The mottling symptoms in the leaves as well as the starch accumulation in the symptomatic transgenic tobacco were accentuated by high light intensity growth conditions. The phenotype observed in the symptomatic, transgenic tobacco associated with impaired photoassimilate partitioning mimic the symptoms induced by geminivirus infections.

Fig. 3-8. Starch-iodine test for starch accumulation.
Newly expanded leaves from transgenic and TMoV-infected (30 days after inoculation) plants were sampled in the morning. The leaves were decolorized in ethanol and stained with iodine-potassium iodide-lactic acid mixture. Note the scattered distribution of starch accumulation in the virus-infected leaf and the uniform distribution in the symptomatic, transgenic tobacco leaf, except for the area near the petiole which was under less light intensity (partially shaded by upper leaves).
(**A**) TMoV-infected (systemic) tobacco leaf
(**B**) non-infected tobacco leaf.
(**C**) symptomatic transgenic tobacco leaf (BC3-11-5S).
(**D**) asymptomatic transgenic tobacco leaf (BC3-11-6A).


A
B
C
D

## Discussion

### Unexpected Phenotype Variations Correlated with Transgene Mutations

The variations in the phenotype in tobacco plants transgenic for the TMoV *BC1* were unexpected since similar levels of the BC1 protein were detected in the asymptomatic as well as symptomatic plants. All of the *BC1* transgenic plants from 6 lines analyzed revealed mutations in the *BC1* gene when compared with the viral sequence and with the sequence from the initial PCR clone used in the gene construction for transformation. Three of the 6 lines analyzed had a single copy of *BC1*. Point mutations were found in all transgenes analyzed and one transgene also showed a major deletion at the 3' end and a recombination event with the addition of ~225 nucleotides (Fig. 3-7A) from an unidentified source. Is this frequency of mutation during plant transformation an unusual occurrence? Is the expression of the wild-type *BC1* ORF lethal to the transgenic cells or are these transgenic cells too slow growing to be selected? All of the nucleotide changes with one exception resulted in an amino acid change. This high correlation of nucleotide changes with amino acid changes may be due to selection involved in mollifying the toxic effects of non-mutated BC1 protein. It has not been resolved whether all the amino acid changes are involved in modifying the BC1 protein in terms of pathogenicity or suppression. All of the amino acid changes resolved in the *BC1* transgenes are in highly conserved regions of the bipartite geminivirus *BC1* gene. The phenotypic variations observed in our TMoV *BC1* transformation appear remarkably similar to those reported for transgenic tobacco expressing SqLCV *BC1* by Pascal et al. (1993), who reported that

5 out of 17 lines expressing SqLCV *BC1* were symptomless, whereas the others showed a range of symptomatic phenotypes. Since they did not report the transgene copy number in their transformants, it is not possible to relate symptom attenuation with transgene interference as shown here for TMoV *BC1* transformants. Their SqLCV *BC1* transgenic lines failed to complement viruses mutated in the *BC1* gene. The failure to complement may be due to mutations in the SqLCV *BC1* gene during transformation.

Transgene *BC1* gene sequence variations other than that seen for *BC1*At/r may result in an asymptomatic phenotype. The *BC1*At/r transgene mutation was only found in one of 19 tobacco lines transgenic for the *BC1* gene. The *BC1* gene sequence resolved from a clone selected from the PCR amplification of a 5 *BC1* gene repeat sequence transgenic plant (BC3-6-3A) revealed changes near the amino terminus (Fig. 3-7). The association of this transgenic phenotype with *BC1* sequence changes was supported by testing the expression of this *BC1*A gene (cloned from BC3-6-3A) in a potato virus X (PVX) expression vector (Chapman et al., 1992). Tobacco inoculated with transcripts of PVX-*BC1* (*BC1* gene derived from TMoV) construct showed veinal necrosis and mottling symptoms characteristic for TMoV while tobacco inoculated with transcripts of the PVX-*BC1*A construct showed very mild mottling symptoms characteristic for the PVX vector infection (Broglio and Powell, unpublished data). Possible variations in the *BC1* gene in the other transgenic lines have not been analyzed because single copy transgene plants have not been identified in these lines.

Deletions, recombination events, and point mutations in non-selected genes during recombinant DNA propagation are rarely reported. Stephens et al (1996) reported that a

mutation in the GUS gene in a clone stock of *A. tumefaciens* LBA4404/pBI121 resulted in loss of GUS activity in transformed tobacco plants. They conclude that non-selected genes within the T-DNA region may undergo mutations in *Agrobacterium.* Donson et al. (1993) observed a 1.4 kbp insert, an IS10-like transposable element, during the propagation of a tobacco mosaic virus (TMV) replicase ORF plasmid. On the other hand, many variations in transgene expression and in the expected transgene phenotypes are reported in studies involving plant virus ORFs. Hong and Stanley (1996) proposed that selection against a functional transgene may have occurred during plant transformation and regeneration to explain the failure of their transgenic tobacco lines for African cassava mosaic virus (ACMV) *AC1* in complementation experiments with ACMV defective in *AC1*. Therefore, spontaneous point mutations in the transgene during *Agrobacterium*-mediated transformation and other modifications in the transgene by chromosomal rearrangements should be considered in the interpretation of gene function and regulation experiments with transgenes.

Transgene Expression Regulation

Other studies on the expression of foreign genes in transgenic plants show various levels of expressivity in different lines or in siblings within a transgenic line (Meyer, 1995). Varying levels of resistance in different transgenic plant lines transformed with the same gene appear to be the norm in pathogen-derived resistance studies (Longstaff et al., 1993; Hull, 1994; de Feyter et al., 1996). Longstaff et al. (1993) found that two lines transgenic for the ADD mutant for PVX replicase component were fully susceptible to PVX even though these lines expressed a higher level of the transgene than a resistant line. de Feyter

et al. (1996) found virus resistance in some transformed tobacco lines that expressed high levels of the transgenes (virus specific ribozyme gene or the antisense construct) while other lines with equivalent or even higher levels of expression showed no detectable virus resistance. Various suggestions to explain the lack of correlation between transgene expression level and the phenotype include somaclonal artifacts, and cell- and temporal-specificities. Other explanations proposed for phenotypic variations in plants transformed with the same gene include epigenetic effects, positional effects due to the random integration in the plant chromosome during transformation, and silencing of the introduced gene (co-suppression, methylation) when multiple copies of the gene are introduced into plant cells (Meins and Kunz, 1995; Hobbs et al., 1990). Hobbs et al. (1993) reported that transgene copy number can be positively or negatively associated with transgene expression, in a study of two different types of T-DNA insert. Their results indicated that co-suppression occurred in multiple gene copy plants only if gene copies were not identical and if the deviant copy resulted in *trans* suppression of the wild-type gene or genes. The *trans* suppression of the "S" phenotype *BC1* gene by the presence of the *BC1*At/f transgene in the "M" phenotype plants in this report shows a similarity to the results reported by Hobbs et al. (1993).

DNA blot analysis of $T_1$ (Fig. 3-3) progeny indicated the "M" phenotype $T_0$ tobacco selected for this study contained two copies of the *BC1* gene. The "M" phenotype plant described above (Fig. 3-2b) apparently had one copy each of the "S" and "A" forms of the *BC1* gene (Figure 3-3, BC3-11-2M). This indicated that the "A" phenotype *BC1* gene suppressed the symptom inducing element(s) of the "S" phenotype

BC1 gene in transgenic plants containing both forms. Apparently $T_0$ transgenic tobacco containing copies of both symptomatic and asymptomatic forms of the *BC1* gene resulted in a phenotype with mild mottling and no stunting. Symptom suppression was also evident in other transgenic lines with multiple *BC1* gene copies since some of these asymptomatic, transgenic $T_0$ tobacco plants also revealed segregation in the $T_1$ and $T_2$ progeny as indicated by the appearance of several symptomatic plants in the progeny. Transgene silencing (Meins and Kunz, 1995) was not evident in the two copy *BC1* plants described above since protein from each copy was detected in protein immunoblots (Fig. 3-4, BC3-11-2M). Furthermore, the expression of the symptomatic phenotype in subsequent generations indicated that the symptomatic *BC1S* gene was active in the asymptomatic phenotype $T_0$ tobacco parent. However, the involvement of other gene regulatory phenomena such as methylation and post-transcriptional modifications (Meyer and Saedler, 1996) could not be excluded in the other lines with multiple *BC1* gene repeats since the level of BC1 protein expression in the calculated 5 repeat transgenic plant (BC3-6-3A) did not appear to be higher than in the single copy transgenic plant (BC3-11-5S) (Fig. 3-4).

Movement Protein Domains

Recent mutagenesis studies on the MP gene of bipartite geminiviruses, bean golden mosaic virus, SqLCV, ACMV, and potato yellow mosaic virus revealed potential functional domains and the tolerance of these viruses to modifications in the MPs (Smith and Maxwell, 1994; Haley et al., 1995; Ingham et al., 1995; Sung & Coutts, 1995). These

mutational analyses, which depended upon virus inoculations with modified infectious clones containing the mutated *BC1* gene, indicated that most mutations at conserved amino acid regions at the N-terminus impaired the function of the MPs but mutations at the C-terminus were less disruptive of function. The pleiotropic nature of the MP (Olesinski et al., 1995) is indicated by a domain that modifies plasmodesmal size exclusion limits and may interfere with endogenous processes involved in regulating photoassimilate movement, a domain involved in symptom induction, and a domain involved in the interaction and cell-to-cell transport of the viral genome. The conserved sequences and motifs involved in geminivirus MP domains remain to be mapped. Another possible domain to be mapped involves the trans-dominant negative interference of the TMoV *BC1At/r* gene on the symptom inducing elements of the *BC1S* gene (BC3-11-2S). The TMoV *BC1* gene mutants identified here provide novel experimental materials for the study of the molecular and physiological mechanisms in symptom induction and attenuation by geminivirus MP.

Symptoms Associated with Starch Accumulation

It has been proposed that virus MPs in plants may significantly disturb source-sink interactions, and reduce photoassimilate partitioning in sink tissues and thus hinder plant growth and development (Leisner and Turgeon, 1993; Lucas et al., 1993). Transgenic tobacco expressing the tobacco mosaic virus (TMV) 30-kDa MP show unexpected interference with carbohydrate metabolism and its export from the mesophyll tissue (Lucas et al., 1993). In a subsequent study with TMV MP mutants, Olesinski et al. (1995)

demonstrate a second function, in addition to the dilation of the plasmodesmata, that involves interaction with cellular processes in carbon metabolism and/or export. The *BC1* transgenic tobacco with the symptomatic phenotype showed starch accumulation, presumably due to impaired photoassimilate partitioning (Fig. 3-8), and mimicked the symptoms induced by geminivirus infections (Fig. 3-2). Lerchi et al. (1995) constructed transgenic tobacco with constitutive, phloem-specific expression of the *E. coli* ppa gene encoding inorganic pyrophosphatase. Removal of the cytosol PPi (pyrophosphate) in the transgenic plants resulted in photoassimilate accumulation in source leaves, chlorophyll loss and stunted growth (reduced internode distance) with no changes in leaf number. Due to the removal of PPi in phloem cells, the export of photoassimilates was inhibited, leading to a large accumulation of starch in leaf mesophyll cells. The tobacco phenotypes with impaired photoassimilate partitioning illustrated by Lerchi et al., (1995) appear remarkably similar to phenotypes seen in some of our transgenic tobacco expressing the *BC1*S gene.

Trans-dominant Negative Interference

Herskowitz (1987) presented the concept of the functional inactivation of a gene by "dominant negative mutation" and described it as the inhibition of the function of a wild-type gene product by an over production of an inhibitory variant of the same gene product. von Arnim and Stanley (1992a) have demonstrated the inhibition of ACMV infection by co-inoculation with a modified version of ACMV genome constructed by replacing the coat protein region with a chimera containing amino-terminal sequences of

TGMV *BC1* and a carboxy-terminal sequence of its ACMV homologue *BC1*. They considered this interference with the function of the wild-type gene as characteristic of a dominant negative mutant. The *BC1*At/r identified in this study was a unique mutation of the TMoV *BC1* gene, which showed trans-dominant interference with the expression of the pathogenicity gene of TMoV. In transgenic tobacco, this mutated BC1 gene not only suppressed the phenotype expression of the symptom-inducing *BC1* gene in the *BC1* transgenic plants but also effectively suppressed the symptoms of TMoV infection (Chapter 4). A number of other transgenic tobacco lines expressing the BC1 protein with an asymptomatic phenotype also showed geminivirus resistance. Presumably these lines also contained the mutated *BC1* gene because of the symptomatic phenotype suppression. These mutated BC1 genes and transgenic plants should enable the studies of the pathogenicity elements of this gene, the studies of the cytological localization and physiological effects of this protein *in planta*, and the identification of the elements in the mutated, transgene *BC1* responsible for the resistance to virus infection.

# CHAPTER 4
# GEMINIVIRUS RESISTANCE IN TRANSGENIC TOBACCO PLANTS EXPRESSING MUTATED BC1 PROTEIN

## Introduction

The recent introduction of a new biotype of the whitefly vector, *Bemisia tabaci* (Gennadius), with a polyphagous nature into the New World (Brown et al., 1995) has resulted in more frequent and more widely distributed geminivirus problems in vegetable and fiber crops. Concurrently, apparently new, whitefly-transmitted geminiviruses have caused serious losses in tomato production worldwide (Navot et al., 1991; Brown & Bird 1992; Kunik et al., 1994; Piven et al., 1995). Tomato mottle virus (TMoV), an example of a new geminivirus pathogen, is a typical bipartite geminivirus (Abouzid et al., 1992b), and was first found in Florida in 1989. The virus was found widespread in Florida's tomato production areas with incidences as high as 95% and with an estimated loss of $125 million in tomato production in southwest Florida in 1990-1991 (Polston et al., 1993). Currently there are no tolerant or resistant cultivars available. The disease control in Florida relies on frequent applications of insecticides including imidacloprid, a systemic insecticide. The potential problem is that the 'B' biotype whiteflies are able to develop insecticide resistance quickly.

Recent advances in molecular biology have enabled the development of "pathogen-derived resistance" where the expression of a pathogen-derived gene/sequence in a plant

has provided for new sources of pathogen resistance (Sanford and Johnson, 1985). Many examples of this type of engineered resistance to RNA plant viruses have been presented (Lomonossoff, 1995; also see Seminars in Virology 4, 1993). The reports, however, for engineered virus resistance in transgenic plants harboring geminivirus DNA sequences are more limited. A number of strategies for pathogen-derived resistance have been investigated for geminivirus resistance. Defective viral DNA, antisense AC1 (replication-associated protein) gene, defective AC1 protein, defective movement protein, and coat protein have been demonstrated to confer geminivirus resistance (Day et al., 1991; von Arnim & Stanley, 1992a; Kunik et al., 1994; Hong & Stanley, 1996). However, unlike the reports for engineered resistance in transgenic plants for RNA viruses, all the transgenic plants reported for geminivirus resistance do not show immune response and conferred resistance is moderate with a narrow spectrum of resistance.

Two movement proteins, BC1 and BV1 (also known as BL1 and BR1 in the literature), encoded by DNA-B of bipartite geminiviruses are required for viral infectivity and systemic infection (von Arnim & Stanley, 1992b; Smith & Maxwell, 1994; Haley, et al., 1995; Ingham et al 1995). The BC1 protein, however, is responsible for cell to cell movement of virus (Noueiry et al., 1994) and is the only determinant of pathogenicity (Pascal et al., 1993; Ingham et al., 1995). In the study on the transformation of tobacco with the TMoV *BC1* gene, three different *BC1* mutants identified from the transgene had marked effects on the phenotype of uninfected transgenic plants (Chapter 3). One asymptomatic *BC1At/r* mutant suppressed the symptom inducing effects of the *BC1S* when both genes were present in the same tobacco line. The symptom suppression by the

*BC1* mutant had characteristics described as "dominant negative interference" (Herskowitz, 1987). In this study, transgenic plants expressing a mutated TMoV BC1 protein with an asymptomatic phenotype show resistance to two distinct geminiviruses.

## Materials and Methods

### Source of Virus, Whiteflies and Inoculation of Plants.

The TMoV and cabbage leaf curl virus (CabLCV) cultures used in this study were the same ones as reported by Abouzid (1992a, 1992b). The inoculations of TMoV and CabLCV were carried out in Gainesville, and Leesburg, Florida, respectively. Whiteflies used in this study came from a colony which has been confirmed as the "B biotype" of *Bemisia tabaci* by isozyme analysis (T. Perring, University of California, Riverside), and were maintained on collards (*Brassica oleracea* var. *acephala*) in an insect-proof greenhouse at CFREC-Leesburg, Florida. For TMoV resistance assessment, whiteflies were reared on tobacco plants (*Nicotiana tabacum* cv. Xanthi) in a greenhouse for several generations, and the TMoV culture in a tomato plant was transmitted to the tobacco plants by whiteflies and maintained on them. Seedlings at the 7-9 leaf stage of transgenic plants selected by germination on kanamycin medium and nontransformed tobacco plants were exposed in cages to about 100 viruliferous whiteflies per plant for 72 hr. The whiteflies on inoculated plants were disturbed once every 24 hr to force movement of the vector among the plants in order to provide a more uniform inoculation. For non-continuous challenge, the whiteflies were killed with insecticide, after inoculation, and plants were transferred into another greenhouse and grown individually in a pot for more

than three months. For continuous challenge, the inoculated plants were kept in the greenhouse for a continuous exposure to viruliferous whiteflies for a period of more than three months with disturbing of the whiteflies every day. The population of viruliferous whiteflies was increased as the challenge continued, resulting in thousands of whiteflies per plant at the end of the test. CabLCV was maintained in cabbage plants. Whiteflies were given an access to CabLCV-infected cabbage plants for a period of 48 hr and then used for inoculating tobacco plants (at the 7-9 leaf stage) for 3-4 days, with approximately 100 whiteflies per plant.

Detection of Virion Antigen and Viral Nucleic Aacids in Plants

Enzyme-linked Immunosorbent assay (ELISA) for the detection of TMoV and CabLCV capsid proteins in tobacco leaf extracts was carried out with the triple-sandwich method as described by Cancino et al. (1995). The wells of ELISA plates (Dynatech Labs, Inc., Chantilly, VA) were coated (150 µl per well) with polyclonal antiserum # 1110 (Cancino et al., 1995) at 1:1000 dilution in coating buffer, pH 9.6, and incubated at 37° C for 1 hr. After incubation, the plates were washed 3 times with PBS-T, pH 7.4. The extracts of leaf samples homogenized at a 1:5 ratio of tissue to PBS, pH 7.2, were added to the wells (150 µl per well) of the antibody-trapped ELISA plates and incubated at room temperature (RT) for 1-2 hr. After washing 5-10 times, 150 µl of 3F7 monoclonal antiserum (Cancino et al., 1995) at 1:10,000 dilution was added to each well of the plates. The plates were incubated at RT for 1 hr or at 4 C for overnight and washed 3 times. Antimouse IgG conjugated with alkaline phosphatase (Sigma Chemical Co., St. Luis, MO)

at 1:30,000 dilution was added to the plates (150 µl per well). The plates were incubated for 1 hr and then washed 3 times. One hundred fifty µl of substrate (1 mg/ml, ρ-nitrophenyl disodium phosphate, Sigma Chemical Co., St. Luis, MO) in substrate buffer (9.7 % diethanolamine, Fisher Scientific, Fair Lawn, NJ) was added to each well of the plates and incubated at RT. Absorbance readings (405 nm) were taken on a Biotek automated microplate reader, model EL 309 (Bio-Tek Instrument Inc. Winooski, Vt) at 1.5-2 hr.

Dot Blot Assay of Viral Nucleic Acid

Total nucleic acids were purified as described in Chapter 3 from the lowermost leaf and uppermost leaves, respectively, at 54 days after inoculation (DAI), and spotted onto H bond $N^+$ nylon membrane (Amersham Life Science Inc.) at 1µg per blot. Viral DNAs were detected by hybridization with $^{32}$ P-labled probe specific to DNA A. The probe was made by random primed $^{32}$P-labeling of CabLCV DNA A with a Prime-a Gene labeling kit (Promega Co., Madison, WI). Hybridization (overnight at 65° C) of blots was in 6 x SSC, 5 x Denhardt's solution and 0.5% SDS, and 0.1 mg /ml salmon sperm DNA. Blots were washed twice in 2 x SSC containing 0.1% SDS at room temperature for 10 min, and once in 1 x SSC containing 0.1% SDS and once in 0.1 x SSC containing 0.1% SDS at 65° C for 15 min (Sambrook et al., 1989).

## Results

### Transgenic Tobacco Expressing BC1 Proteins

High levels of BC1 protein were detected in all $T_1$ lines except one (BC3-11-6) which showed a low level of a smaller (23 kDa versus the expected 33 kDa) protein (Fig. 4-1). This *BC1* transgene (*BC1*At/r; Genbank #U65506) had undergone a recombinational event which resulted in an open reading frame with a deletion of 119 carboxy-terminal amino acid residues and an addition of 26 amino acids from an unknown source (Chapter 3). The low level of BC1 protein detected in the BC3-11-6 line may be due to the loss of epitopes since more than 1/3 of the codons for carboxy-terminal amino acid residues were lost during the recombinational event (Chapter 3).

### Transgenic Tobacco Plants Resistant to TMoV

Based on the asymptomatic phenotypes and virus resistance in $T_0$ transgenic plants (data not shown), selected transgenic tobacco lines ($T_1$ generation) at the 7-9 leaf stage were exposed to viruliferous (TMoV) whiteflies for a 72 hr inoculation exposure in a greenhouse (Table 4-1). All of the transgenic lines tested, except certain ones expressing mutated BC1 protein, were highly susceptible to TMoV. Based on this test and others (data not shown) we focused our analysis of virus resistance on three *BC1* transgenic lines, BC-3-6, BC-3-11 and BC-3-31. The transgenic tobacco plants ($T_1$ and $T_2$ generations) carrying mutated BC1 genes showed the following symptoms after inoculation:

1. No visible symptoms throughout the life of the plants, indistinguishable from noninoculated plants (Fig. 4-2 D and on the right in A).

2. Marked delay of symptoms for several weeks after exposure to viruliferous whiteflies with new growth showing typical TMoV symptoms (Fig. 4-4).

3. Symptoms of vein chlorosis and mottling on early leaves after exposure to viruliferous whiteflies but subsequent growth with significant remission of symptoms, consistent with significant reduction of virions (Fig. 4-3).

4. Stunting, mottling, and vein chlorosis on the leaves, typical symptoms associated with TMoV infections in nontransformed tobacco plants (Fig. 4-2 C and on the left in A).

In general, the leaves exposed to viruliferous whiteflies (72 hr inoculation exposure) in most of the transgenic plants expressing mutated BC1 protein(s) showed no symptoms after the plants were challenged with TMoV (Table 4-1 and 4-2). Some of these plants initially showed disease symptoms similar to infected nontransformed plants, but subsequent growth plants showed remission of symptoms, and ELISA tests indicated the reduction of TMoV levels in these plants (Fig. 4-3). Such a recovery phenomenon has been reported in other studies with transgenic plants expressing viral sequences (reviewed by Lomonossoff, 1995). Under continuous inoculum pressure and extreme temperature conditions (non air-conditioned greenhouse, temperatures often exceeded $40^0$ C daily) a number of $T_2$ generation lines showed an apparent delay in symptom development (Fig. 4-4). In addition, many of the progeny of transgenic tobacco line (BC3-11-6) expressing mutated BC1 protein (*BC1*At/r) appeared free of visible symptoms throughout the three

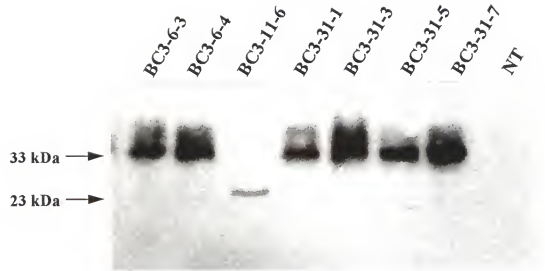



Fig. 4-1. Western blot analysis of transgenic $T_1$ tobacco plants expressing the *BC1* gene. Extracts from young leaf tissue were fractionated as described in Material and Methods in Chapter 3. Eight microliters of the P30 fractions from transgenic lines were electrophoresed on SDS-PAGE, and transferred to nitrocellulose membrane. The BC1 protein was detected with the anti-BC1 polyclonal antiserum. The full length and the truncated BC1 proteins are indicated with approximate sizes. Lanes representing extracts from transgenic plant lines with asymptomatic phenotypes and nontransformed control plant (NT) are noted.

Fig. 4-2. Resistance of $T_2$ transgenic tobacco plants to TMoV and CabLCV infections. Transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi) expressing the BC1 proteins (Fig. 4-1) were challenged with TMoV- or CabLCV-viruliferous whiteflies. The nontransformed plants (C and on the left in A) and susceptible transgenic plant (on the left in B) show typical disease symptoms of TMoV and CabLCV infection, respectively, whereas the resistant transgenic plants are free of disease symptoms ( D, and on the right in A and B).

A) Plants were exposed to TMoV-viruliferous whiteflies for 72 hr at the 9 leaf stage. The plant on the left is a nontransformed plant, and the plant on the right is the progeny of BC3-11-6. Photographed 50 days after exposure to viruliferous whiteflies.

C) and D). Nontransformed plant (C) and transgenic plants (D, progeny of BC3-11-6) were continuously challenged with TMoV-viruliferous whitefles for a three month period. The photos were taken at 80 days after the start of the exposure to viruliferous whiteflies. Arrows indicate the whitefly density at the late stage during the challenge period.

B) Plants were exposed to CabLCV-viruliferous whiteflies for 72 hr at the 7-9 leaf stage. The plant on the left is the progeny of BC3-6-3, while the plant on the right is the progeny of BC3-11-6. Photographed 40 days after exposure to viruliferous whiteflies.

A

B

C

D

Fig. 4-2--continuted

Table 4-1. Assessment of TMoV resistance in $T_1$ transgenic plants with different constructs.

| Plant lines | No. plants diseased/inoculated[1] (No. expts.) | ELISA value[2] of diseased/asymptomatic plants |
|---|---|---|
| Nontransformed | 32/34 (6) | 0.665/0.008 |
| BC3-6 | 3/20 (4) | 0.323/0.011 |
| BC3-11 | 25/32 (6) | 0.583/0.010 |
| BC3-31 | 9/30 (4) | 0.492/0.007 |
| BVas2-1[3] | 10/10 (2) | 0.595/-- -- |
| BVas2-6 | 7/9 (2) | 0.603/0.013 |
| BV9-15 | 15/15 (2) | 0.626/-- -- |
| BCas8-8 | 12/12 (2) | NA[4] |
| BCas8-11 | 14/14 (2) | NA |
| BCas8-14 | 12/16 (2) | NA |
| BV3-9 | 15/15 (2) | NA |
| BV9-1 | 14/14 (2) | NA |
| BV9-7 | 16/16 (2) | NA |

[1] The transgenic (resistant to kanamycin) and control plants at the 7-9 leaf stage were exposed in a cage to viruliferous whiteflies for 72 hr, with approximately 100 whiteflies per plant.

[2] Indirect ELISA was used to assay the inoculated plants at 35-40 days after inoculation (DAI). The polyclonal antiserum 1110 and monoclonal antiserum 3F7 were used in the indirect ELISA as described in Material and Methods. Newly expanded leaves from individual plants were collected, and homogenized in 1:5 (w/v) PBS for the assay. The ELISA readings represent the mean values ($A_{405}$) from three duplication reactions of all diseased plants or all resistant plants (asymptomatic) in a given group. In all cases, a positive ELISA result was correlated with the appearance of viral symptoms.

[3] as = antisense oreintation of the gene.

[4] NA= not analyzed.

Table 4-2. Assessment of TMoV resistance in $T_2$ transgenic plants expressing the *BC1* ORF

| Plant lines[1] | No.transgene copy | No. plants diseased/inoculated | ELISA value[2] of diseased/asymptomatic plants |
|---|---|---|---|
| Nontransformed | 0 | 6/6 | 0.642/-- -- |
| BC3-6-4 | 3 | 1/6 | 0.327/0.012 |
| BC3-11-6 | 1 | 3/7 | 0.347/0.008 |
| BC3-31-1 | 3 | 1/6 | 0.969/0.011 |
| BC3-31-5 | 1 | 0/7 | -- --/0.009 |
| BC3-31-7 | 4 | 2/6 | 0.782/0.010 |

[1] The transgenic (resistant to kanamycin) and control plants at the 7-9 leaf stage were exposed in a cage to viruliferous whiteflies for 72 hrs, with approximately 100 whiteflies per plant. Plant line and copy number refer to $T_1$ generation, with the exception that the copy number for BC3-31-5 was identified in one of the $T_2$ progenies.

[2] Indirect ELISA was used to assay the transgenic plants 40 days after inoculation. as described in Table 1.

Table 4-3. Assessment of CabLCV resistance in $T_2$ transgenic plants expressing the *BC1* mutants

| Plant lines | No.transgene copy | No. plants diseased/inoculated[1] | ELISA value of diseased / asymptomatic plants |
|---|---|---|---|
| BC3-6-3 | 5 | 4/5 | 0.786/0.007 |
| BC3-6-4 | 3 | 2/8 | 0.690/0.010 |
| BC3-11-6 | 1 | 3/8 | 0.617/0.009 |
| BC3-31-5 | 1 | 1/9 | 1.065/0.013 |
| BC3-31-7 | 4 | 0/5 | -- --/0.008 |

[1] Transgenic plant seedlings resistant to kanamycin (see Table 1) were exposed in a cage to viruliferous whiteflies for 72 hr, with approximately 100 whiteflies per plant at the 7-9 leaf stage. Plant line and copy number refer to $T_1$ generation, with the exception that the copy number for BC3-31-5 was identified in one of the $T_2$ progenies. At 52 days after inoculation, indirect ELISA was used to assay transgenic plants as described in Table 1.

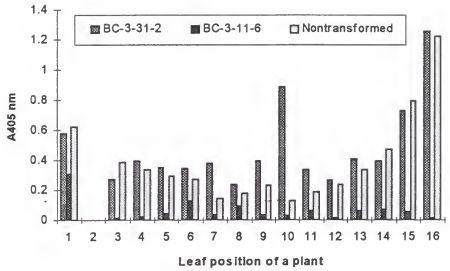



Fig. 4-3. Recovery phenomenon in TMoV-infected transgenic plants expressing BC1At/r mutant (BC3-11-6).
Plants were inoculated as indicated in Table 1. TMoV concentration in infected plants was analyzed by ELISA as described in Table 1. No. 1 represents ELISA mean values of first test with the three different groups of infected plants (two plants each) at 21 days after inoculation (DAI). No. 3 to 16 represent ELISA mean values of second test of the different leaves starting from the oldest leaf (3) to the youngest newly expanded leaf (16) with sampling every third leaf of the infected plants at 85 DAI.

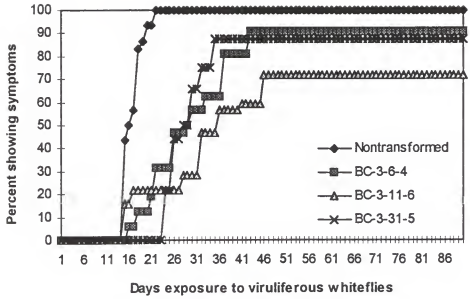



Fig. 4-4. Assessment of TMoV resistance in $T_2$ transgenic tobacco plants expressing BC1 mutants.

Transgenic (resistant to kanamycin) and nontransformed tobacco seedlings (32 plants per line) at the 7-9 leaf stage were exposed in a greenhouse to viruliferous whiteflies for a period of more than 3 months (March to June). The whiteflies were raised on infected tobacco plants in the greenhouse, and multiplied to a large population during the challenge period, which resulted in about a hundred whiteflies at the begining to thousands of whiteflies per plant at the end. The percentage of plants in each transgenic plant line that had systemic disease symptoms is plotted against days of exposure to viruliferous whiteflies.

month exposure to thousands of viruliferous (TMoV) whiteflies (Fig. 4-2 D). ELISA tests of these virus challenged, transgenic tobacco plants indicated undetectable to very low levels of TMoV (data not shown). Positive readings (3 x over background) always corresponded to disease symptoms in the ELISA tests of more than 300 samples.

Some of the variation in resistance (Fig. 4-4) to TMoV in the transgenic tobacco may be attributed to the segregation of the multiple transgene copies. Selected $T_1$ generation lines tested for resistance were analyzed by Southern blots for transgene copy number (Table 2). However, it was not resolved whether resistance of the lines with a single copy of *BC1* transgene was associated with homozygous or heterozygous states. Highly TMoV-resistant transgenic plants were found among the single or multiple copy *BC1* transgenic plants.

Resistance to another geminivirus

The $T_2$ progenies from highly TMoV resistant $T_1$ lines were tested against a cabbage leaf curl geminivirus (CabLCV, Genbank #U65529 and U65530), a virus which shares about a 70% nucleotide sequence similarity to TMoV. All the inoculated plants showed mild symptoms at the inoculated leaves, presumably due to whitefly feeding damage and to virus infection (chlorotic spots). Many of the resistant plants subsequently grew free of symptoms (Fig. 4-2 B; Table 4-3) while others showed a significant delay (2-5 weeks) in symptom development. The level of the CabLCV in the test plants was evaluated by ELISA (Table 4-3) and dot blot analysis (Fig. 4-5). The plants free of virus symptoms had ELISA readings below the detection level for virus. Plant lines showing

high levels of resistance to TMoV (Table 4-2) also showed similar levels of resistance to CabLCV (Table 4-3). The dot blot analysis involved separate sampling and testing of leaves including the inoculated leaves. The inoculated leaves tested positive in most cases, indicating that virus replication occurred in these leaves while upper leaves for the plants free of systemic symptoms tested negative (Fig. 4-5). The mutated *BC1*-mediated resistance may involve the suppression of systemic movement of the virus. The resistance to CabLCV indicated that certain transgenic lines expressing mutated BC1 protein may have a broad spectrum of resistance to bipartite geminiviruses.

## Discussion

### Geminivirus Resistance

The spontaneously mutated *BC1* transgene (*BC1*At/r) was shown previously (Chapter 3) to suppress the symptom inducing effects of the *BC1* gene in transgenic plants containing one copy of both genes. Here we show that the same transgenic tobacco with the *BC1*At/r (BC3-11-6) also provides resistance to infections by two distinct geminviruses, TMoV and CabLCV. This mutant *BC1*A t/r was found in only one of the 19 lines transformed with *BC1*. Presumably the other lines BC3-31-x and BC3-6-x may contain other form(s) of spontaneously mutated *BC1* transgene (Chapter 3) since they also show geminivirus resistance while expressing high levels of BC1 protein (Fig. 4-1). The variation of resistance may be associated with the heterozygous or homozygous state of the transgene in plants of the single copy line (3:1 segregation on kanamycin selective

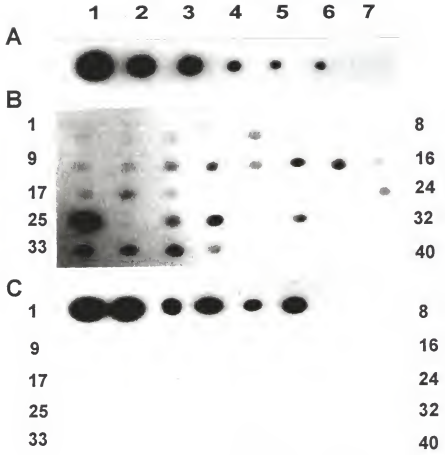
Fig. 4-5. Dot blot analysis of CabLCV DNA in transgenic tobacco plants at 54 days after inoculation.

Total nucleic acids were purified from inoculated and noninoculated plants, and were spotted onto H bond $N^+$ membrane at 1 μg per blot (except for samples on A). Viral DNAs were detected by $^{32}P$-labeled probe specific to CabLCV DNA.

(A) Lane, 1, 1 μg of total nucleic acid from CabLCV-infected cabbage; Lanes 2-6, serial dilution of 1:5, 1:10, 1:50, 1:100 and 1:100, respectively. The autoradiograph was developed after a 2 hr exposure.

(B) Samples from lowest leaves of noninoculated control plants are shown in spot Nos. 38, 39 and 40. All other spots represent samples from inoculated transgenic plants, which showed whitefly feeding damage and white spot symptoms, presumably due to CabLCV infection. Samples spotted on Nos. 12, 14, 15, 17, 24, 25, 28, 30, 33 and 34 are from plants which showed systemic symptoms. None of the other plants showed systemic symptoms. The autoradiograph was developed after a 5 day exposure.

(C) Samples from upper leaves of plants as in B. Spot Nos 1-6 from plants with systemic symptoms, which were the same as those spotted on Nos. 33, 28, 17, 15, 14, and 12 as in B. Samples spotted on Nos. 7 to 37 represent plants starting at 1 as in B except for those plants showing systemic symptoms listed in B. The autoradiograph was developed after a 2 hr exposure.


1 2 3 4 5 6 7
A
B
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medium) and the segregation of the lines with multiple transgene copies (varied segregation on the selective media; data not shown). Further genetic and biochemical dissections of transgene copies in these lines may resolve the potential correlation of the mutated versus nonmutated transgenes.

Viral resistance tests with TMoV were done under a range of conditions. The resistance experiments involving continuous inoculation pressure were done with numerous whiteflies per plant (Fig. 4- C and D) and the whiteflies were forced to move about the test plants by shaking the leaves of the test plants once a day. In addition one round of testing was done during March to June in a greenhouse where the temperature often exceeded 40° C midday during May to June. The whiteflies thrived under these conditions and susceptible plants showed full-blown TMoV symptoms, indicating that the high temperatures did not result in heat therapy. The plants under these conditions showed heat stress and damage due to the extensive whitefly feedings. In order to avoid these complications in evaluating geminivirus resistance, other resistance tests were done with a limited 72 hr inoculation period with viruliferous whiteflies (~100 per plant) which were adapted to tobacco and were done under more moderate temperatures (air-conditioned greenhouse). Bedford et al. (1994) tested the geminivirus transmission efficiencies of the "B" biotype whitefly and found that 20 insects per plant resulted in 100% virus transmission in most tests. S. Webb and J. E.Polston (unpublished data) found that 40 viruliferous whiteflies per plant resulted in a 100% infection rate with TMoV. A number of plant lines showed resistance under a range of inoculation tests and under conditions exceeding the inoculation pressure expected in the field.

The resistance of certain lines to CabLCV is remarkable since this virus is quite different from TMoV. Sequence comparisons of the A component DNA for New World" bipartite geminivruses show CabLCV closely related to SqLCV, tomato golden mosaic virus, and bean golden mosaic virus (Brazil isolate) and distantly related to TMoV and similar geminiviruses like abutilon mosaic virus, potato yellow mosaic virus, and sida mosaic virus (Hiebert et al., 1996). TMoV has a narrow host range (Polston et al., 1993) while CabLCV has a very broad host range (Strandberg et al., 1991). The symptoms of CabLCV infections in tobacco are much more severe (Fig. 4-3 B) than those induced by TMoV.

Many of the pathogen-derived resistance studies (Lomonosoff, 1995) show high virus specificity with the exception of studies involving viral movement protein (MP) (Lapidot et al., 1993; Beck et al., 1994; Cooper et al., 1995). Lapidot et al. (1993) demonstrated that a mutant TMV MP gene in transgenic tobacco showed resistance to virus spread when inoculated with TMV or distantly related tobamoviruses. Beck et al. (1994) found that transgenic tobacco which expressed a mutated white clover mosaic potexvirus (WClMV) 13 kDa gene within a triple gene block were resistant to WClMV and two other members of the genus (potato virus X and narcissus mosaic virus) and to potato carlavirus S. All these viruses have a triple gene block that codes for proteins necessary for cell-to-cell movement. Cooper et al. (1995) report tobacco transgenic for defective TMV MP show resistance to six taxonomically distinct RNA viruses. The resistance results with mutated TMoV BC1 proteins extend the possibility of multivirus resistance to DNA plant viruses using mutated MP genes.

Theory of Resistance

The virus resistance conferred by expression of the mutated *BC1* protein(s) in the transgenic lines may be due to trans-dominant, negative interference (Herskowitz, 1987). This type of resistance may depend upon a relatively high level of expression of the mutated transgene. The low level of BC1At/r protein detection for BC3-11-6 may be due to loss of epitopes since the level of steady state transcript for this gene was similar to the other *BC1* expressing lines (Chapter 3). The resistance may be protein-mediated. It is proposed that the mutated BC1 protein competes for a site or sites involved in the function of wild-type viral protein, and disrupts an important process in the life cycle of the virus. One of the functions of the MP involves interaction(s) with host components since BC1 protein increases the mesophyll plasmodesmal size exclusion limit (SEL) (Noueiry et al., 1994). Presumably, different viruses utilize the same host components for cell-to-cell movement since an increase in plasmodesmal SEL is a common phenomenon associated with plant virus MPs (Lucas & Gilbertson, 1994). Therefore any interference with normal virus-host component interaction should result in a broad spectrum of virus resistance. Other viral genes, especially those involved in viral replication, tend to be very virus specific and thus any interference at this level also results in narrow spectrum virus resistance (Lomonossoff, 1995).

Resistance to RNA Viruses?

Complementation of apparent movement function in RNA and DNA viruses is possible in heterologous combinations as seen in mixed virus infections of bean golden

mosaic virus (BGMV) and TMV (Carr and Kim, 1983). In a preliminary study, the selected *BC1* transgenic lines from the $T_1$ generation were tested for resistance to cucumber mosaic virus (CMV), tobacco etch virus, potato virus Y, and TMV by mechanical inoculation with extracts from infected plants. Transgenic (*BC1*) tobacco plants with the asymptomatic phenotype showed reduced TMV disease symptoms compared to inoculated nontransgenic plants (data not shown), whereas the *BC1* transgenic tobacco with a symptomatic phenotype appeared to accentuate the symptoms induced by TMVor CMV. This is similar to the the report of Cooper et al. (1995) who found transgenic tobacco for wild-type TMV movement protein accentuated the severity of related or unrelated virus infections. None of the transgenic plants showed any resistance to potyviruses, TEV and PVY.

Strategy for the development of geminivirus resistant plants

*Agrobacterium*-mediated transformation with pathogenicity genes may result in transgene mutations and a selection for regenerated plants with modified/suppressed forms of the pathogenicity gene. For geminiviruses, the introduction of the pathogenicity gene (*BC1* for the bipartite geminiviruses, *AC4* for the monopartite geminiviruses like tomato yellow leaf curl virus, Rigden et al., 1994) into plant cells by *Agrobacterium*-mediated transformation may result in selection since transformed cells which express the non-mutated pathogenicity gene will not grow as well as those cells which express the mutated pathogenicity gene. This phenomenon has been observed or implied in transformation with the *BC1* gene of two different bipartite geminiviruses (Pascal, et al. 1993, Chapter 3).

Using this approach, one may readily identify the naturally occurring mutants during transformation, which may confer virus resistance.

# CHAPTER 5
# SUMMARY AND CONCLUSIONS

The *AC3*, *AC4*, *BC1* and *BV1* open reading frames encoding for nonstructural proteins of tomato mottle virus (TMoV) were individually cloned into the pETh3C expression vector and expressed in *Escherichia coli*. The expressed proteins were purified from the cell lysates and were used as immunogens for polyclonal antiserum production in rabbits.

The antisera were used in Western blot analysis to index the expression of the nonstructural genes, *AC3*, *AC4*, *BC1* and *BV1* in TMoV-infected and transgenic plants. The BC1 movement protein (MP) (33-kDa) was detected primarily in the membrane fraction and in the cell wall fraction of leaf extracts from TMoV-infected and transgenic plants. The BV1 MP (29-kDa) was detected in the soluble fraction and primarily in the membrane fraction of the leaf extracts of infected plants. The AC3 (16-kDa) and AC4 protein (10-kDa) were also detected in the membrane fraction of leaf extracts from transgenic plants and/or infected plants. These results are similar to those reported for bipartite geminiviruses, tomato golden mosaic virus (TGMV), squash leaf curl virus (SqLCV) and African cassava mosaic virus (ACMV) (Von Arnim et al., 1993, Pascal et al., 1993; Pedersen & Hanley-Bowdoin, 1994). However, It is the first time that the AC4 protein was detected in the extracts of tomato plants infected with a bipartite geminivirus.

Although both the TMoV MPs can be detected in the extracts from infected plants, the BV1 MP appeared to accumulate as leaves aged in contrast to the BC1 MP. Both MPs have been found to be phosphorylated *in vivo* (Pascal et al., 1994), but only the BC1 protein of bipartite geminiviruses shows an anomalous behavior.

Transgenic tobacco plants were generated by *Agrobacterium*-mediated transformation with the *AC4*, *BC1* and *BV1* gene constructs to study their gene function and potential utilization for virus resistance. The DNA fragments for each of these genes were amplified by PCR from extracts of TMoV-infected plants, and cloned into the expression vector pKYLX71:35S[2] to generate both antisense and/or sense constructs of these genes. The clones of these constructs were immobilized into the cells of an *Agrobacterium tumefaciens* strain, and then the *Agrobacterium* cultures were used to transform the leaf discs of *Nicotiana tabacum* cv. Xanthi. A total of 132 plants generated from the transformations were screened by PCR using specific primers for each of these genes and by ELISA tests using a NPTII ELISA kit. Nineteen *BC1* sense, 16 *BC1* antisense, 20 *BV1* sense dimer, 7 *BV1* sense, 5 *BV1* antisense, and 21 *AC4* sense transgenic plants were obtained. All these transgenic plants were self-pollinated to produce the $T_1$ generation, and selected $T_1$ lines were propagated for the $T_2$ generation.

Transgenic plants expressing BC1 protein had phenotypes ranging from severe stunting and leaf mottling to no visible symptoms. Eight of the 19 transgenic To plants which expressed BC1 protein did not show any virus-like symptoms. The variations in the phenotype of transgenic tobacco plants were unexpected since similar levels of the BC1 mRNA and protein (with the exception for the truncated one from BC3-11-6 line) were

detected in the asymptomatic as well as symptomatic plants. The sequence data for the *BC1* transgene from the different phenotypes indicated at least three different mutated forms of the *BC1* gene in the transformed tobacco. All of the *BC1* transgenic plants from 6 lines analyzed revealed mutations in the *BC1* gene when compared with the viral sequence and with the sequence from the initial clone of PCR products used in the gene construction for transformation. Point mutations were found in all transgenes analyzed, and a recombination was found in one transgene. This *BC1*At/r had a modified *BC1* open reading frame with a deletion of 119 amino acid residues at carboxy-terminus and an addition of 26 amino acids from an unknown source. All the nucleotide changes with one exception resulted in an amino acid change. This high correlation of nucleotide changes with amino acid changes may be due to selection involved in mollifying the toxic effects of non-mutated BC1 protein. The phenotype variations observed in the *BC1* transgenic plants appear remarkably similar to those reported for transgenic tobacco expressing SqLCV *BC1* by Pascal et al. (1993), who reported that 5 out of 17 lines expressing SqLCV *BC1* were symptomless, and the others showed a range of symptomatic phenotypes. Our results suggest that spontaneous point mutations in the transgene during *Agrobacterium*-mediated transformation and other modifications in the transgene by chromosomal rearrangements need to be considered in the interpretation of gene function and regulation experiments.

The transgenic tobacco line (BC3-11) with the mild ("M") phenotypes contained two copies of the *BC1* gene idenfied by Southern blot analysis. Three different phenotypes were found to be associated with the transgene segregation of this line in $T_1$

progenies. The "M" phenotype plant had one copy each of the severe ("S") (*BC1S*) and asymptomatic ("A") (*BC1At/r*) forms of the *BC1* gene. This may be explained that the *BC1At/r* suppressed the symptom inducing effects of the *BC1S* in transgenic plants containing both of these genes. Symptom suppression was also evident in other transgenic lines with multiple *BC1* gene copies since some of these asymptomatic, transgenic $T_0$ tobacco plants also revealed segregation in the $T_1$ and $T_2$ progeny as indicated by the appearance of several symptomatic plants in the progeny. The expression of the symptomatic phenotype in subsequent generations indicated that the symptomatic *BC1*S gene was active in the asymptomatic phenotype $T_0$ tobacco parent. However, the involvement of other gene regulatory phenomena such as methylation and post-transcriptional modifications (Meyer & Saedler, 1996) could not be excluded in the other lines with multiple *BC1* gene copies since the level of BC1 protein expression in the calculated 5 copy transgenic plant did not appear to be higher than in the single copy transgenic plant.

The *BC1* transgenic tobacco with the symptomatic phenotype mimicked the symptoms induced by geminivirus infections, and showed starch accumulation by iodine staining, presumably due to impaired photoassimilate partitioning. It has been proposed that virus MPs in plants may significantly disturb source-sink interactions, and reduce photoassimilate partitioning in sink tissues and thus hinder plant growth and development (Leisner and Turgeon, 1993; Lucas et al., 1993).

Transgenic plants were evaluated for resistance to several viruses including TMoV, cabbage leaf curl virus (CabLCV), tobacco mosaic virus (TMV), cucumber

mosaic virus (CMV), tobacco etch virus (TEV), and potato virus Y (PVY) by inoculation with viruliferous whiteflies and mechanical inoculation. All of the transgenic lines tested, except certain ones expressing mutated BC1 protein, were highly susceptible to TMoV. Three of the BC1 transgenic lines were selected and were intensively tested for geminivirus resistance. Plants expressing the *BC1* mutants with asymptomatic phenotypes were found to be resistant to TMoV and CabLCV.

In general, the leaves exposed to viruliferous whiteflies in most of the transgenic plants expressing mutated BC1 protein(s) showed no symptoms after the plants were challenged with TMoV. Some of these plants initially showed disease symptoms similar to infected nontransformed plants, but subsequent growth showed remission of symptoms, and ELISA tests indicated the reduction of TMoV levels in these plants. Such a recovery phenomenon has been reported in other studies on transgenic plants expressing viral sequences (reviewed by Lomonossoff, 1995). Under continuous inoculum pressure and extreme temperature conditions (non air-conditioned greenhouse in which temperatures often exceeded $40^0$ C daily) a number of $T_2$ generation lines showed an apparent delay in symptom development. In addition, many of the transgenic tobacco plants (BC3-11-6) expressing mutated BC1 protein (BC1At/r) appeared free of visible symptoms throughout the three month exposure to thousands of viruliferous (TMoV) whiteflies. These virus-challenged, transgenic plants had undetectable or very low levels of TMoV in ELSIA tests. It is noteworthy that positive ELISA readings (3 x over background) were always correlated with virus symptoms.

The $T_2$ progenies from highly TMoV resistant $T_1$ lines were tested against CabLCV, a geminivirus which shares about a 70% nucleotide sequence similarity to TMoV. All the inoculated plants showed mild symptoms in the inoculated leaves, presumably due to whitefly feeding damage and to virus infection (chlorotic spots). Many of the resistant plants subsequently grew free of symptoms, while others showed a significant delay (2-5 weeks) in symptom development. The levels of the CabLCV in the inoculated plants were evaluated by ELISA tests and dot blot analysis. The plants without visible virus symptoms had ELISA readings below the level of detection for virus. Plant lines showing high levels of resistance to TMoV also showed similar levels of resistance to CabLCV. The dot blot analysis involved separate sampling and testing of leaves including the inoculated leaves. The inoculated leaves tested were positive in most cases, indicating that virus replication occurred in these leaves. Whereas upper leaves tested were negative in the plants free of systemic symptoms,. These results suggest that the mutated *BC1* mediated resistance may involve the suppression of systemic movement of the virus. The resistance to CabLCV indicated that certain transgenic lines expressing mutated BC1 protein may have a broad spectrum of resistance to bipartite geminiviruses.

Some of the variation in resistance in the transgenic plants may be attributed to the segregation of the multiple transgene copies. It was not resolved whether resistance of the lines with a single copy of the *BC1* transgene was associated with homozygous or heterozygous states. Highly resistant transgenic plants were detected among the transgenic tobacco plants harboring single or multiple copies of the *BC1*.

Transgenic tobacco plants with the asymptomatic phenotype showed reduced TMV disease symptoms compared to inoculated nontransformed plants, whereas transgenic tobacco with symptomatic phenotype appeared to accentuate the systemic infection of TMV or CMV. This is similar to the report of Cooper at al. (1995) who found transgenic tobacco expressing wild-type TMV movement protein accentuated the severity of related or unrelated virus infections.

The resistance conferred by the expression of the mutated *BC1* in transgenic tobacco may involve trans-dominant, negative-interference (Herskowitz, 1987). The *BC1*At/r in transgenic plants not only suppressed the phenotype expression of the symptom-inducing *BC1S* gene in transgenic plants but also effectively suppressed the symptoms of TMoV infection. A number of other transgenic tobacco lines expressing the BC1 protein with an asymptomatic phenotype also show geminivirus resistance. Presumably these lines also contain the mutated *BC1* gene because of the symptomatic suppression. These results suggest that the mutated BC1 protein competes for a site or sites involved in the function of wild-type viral protein, and disrupts an important process in the life cycle of the virus.

*Agrobacterium*-mediated transformation with a pathogenicity gene may result in transgene mutation(s) and a selection for regenerated plants with modified forms of the pathogenicity gene. For geminiviruses, the introduction of the pathogenicity gene (*BC1* for the bipartite geminiviruses, *AC4* for the monopartite geminiviruses such as tomato yellow leaf curl virus, Rigden et al., 1994) into plant cells by *Agrobacterium*-mediated transformation may result in a selection since transformed cells which express the non-mutated pathogenicity gene will not grow as well as those cells h express the mutated one.

Using this approach, one may readily identify the naturally occurring mutants during transformation, which may confer virus resistance.

The spontaneously mutated TMoV *BC1* identified in this study should provide a basis for further studies on the pathogenicity elements of this gene, on the cytological localization and physiological effects of this protein *in planta*, and on the elements of the mutated, transgene *BC1* responsible for the resistance to virus infection, and most importantly, they may be used as novel resistance genes for the control of geminivirus diseases.

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## BIOGRAPHICAL SKETCH

Yong-Ping Duan was born in Fujian, P.R.China, on December 16, 1956. He graduated from Fujian Agricultural University in 1979 with major in Plant Protection. He was a teacher in Longyan Agricultural College in 1979-1980. In 1980-1983, he went to graduate school at Hunan Agricultural University, and majored in Plant Pathology. He got his M.S. from Nanjing Agricultural University in 1985, after which he started his faculty position in the Virology Institute, Fujian Agricultural University. He went to the International Rice Research Institute as a Research Fellow in 1987-1988. He came to the Department of Plant Pathology, University of Florida as a Visiting Scientist, working under Dr. E. Hiebert's supervision in 1991. He began his Ph.D. graduate program in 1993 under the supervision of Drs. E. Hiebert and C.A. Powell, working on molecular biology of tomato mottle virus and the development of transgenic plants resistance to geminiviruses. He expects to complete this program in August and receive his Ph.D. degree in December, 1996. Yong-Ping Duan is a member of the American Phytopathological Society.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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